HOW MULTIPLE PARTITIVIRUSES TRANSFER TO NEW *HETEROBASIDION* HOSTS AND AFFECT THEIR GROWTH

Ping Xu

Master's thesis

Plant and Forest Biotechnology

University of Helsinki

Department of Agricultural Sciences

February 2019

HELSINGIN YLIOPISTO — HELSINGFORS UNIVERSITET — UNIVERSITY OF HELSINKI

Tiedekunta/Osasto — Fakultet/Sektion — Faculty	Osasto — Sektion — Department
Faculty of Agriculture and Forestry	Department of Agricultural Sciences

Tekijä — Författare — Author

Ping Xu

Työn nimi — Arbetets titel — Title

How multiple partitiviruses transfer to new *Heterobasidion* hosts and affect their growth

Oppiaine —Läroämne — Subject

Plant and Forest Biotechnology

Työn laji — Arbetets art — Level	Aika — Datum — Month and year	Sivumäärä — Sidoantal — Number of pages
M.Sc. Thesis	February 2019	100

Tiivistelmä — Referat — Abstract

Heterobasidion is a conifer pathogen that causes tremendous economic loss across the northern hemisphere. It is a root rot pathogen and *Heterobasidion* infected wood can no longer be used as timber. On spruce the decay is usually observed only in the final cutting, on pines it is visible earlier as the tree dies. It is a worldwide pathogen that is quite common in Europe, Asia and north America. Scientists have yet to find an effective way to treat this disease. The use of viruses against *Heterobasidion* as a biocontrol method has shown new promise to cure it. In nature, *Heterobasidion* partitivirus stays inside their host fungus and often shows no symptoms. It was believed before that each type of partitivirus has its own host *Heterobasidion* species and does not transfer to other *Heterobasidion* species. However, previous studies have shown that partitivirus is able to transfer to new *Heterobasidion* host in laboratory and in field, and surprisingly can cause growth rate decrease in its new host.

Researchers have previously studied the effects of many partitivirus species on *Heterobasidion*, but they have focused on analyzing the effects caused by only one virus strain at a time. This thesis focuses on studying the transmission of multiple partitiviruses to a new *Heterobasidion* host, and on understanding how mixed partitivirus infections affect the growth rate of their new host. To study the transmission of many viruses, two different fungus species both hosting five different partitiviruses were selected as donor fungus strains, and ten different virus-free *Heterobasidion* fungus strains were selected as recipient fungus strains. Then growth rate experiment was conducted to figure out whether and how multiple viruses infection affect the growth rate of *Heterobasidion*.

The results of this thesis show: 1) It is possible for multiple partitiviruses to transfer to new *Heterobasidion* hosts. Among 40 transmission experiments, in 13 experiments the recipients were successfully infected by two or more partitiviruses and in 9 experiments the recipients were successfully infected by three or more partitiviruses. 2) Partitiviruses transmission from *H. parviporum* to *H. annosum* is considerably less effective than from *H. parviporum* to *H. parviporum*. This enlightens us that partitiviruses are easier to be transmitted within species border than across species border. 3) Growth rate experiments showed that multiple virus infection has diverse effects on the growth rate of *Heterobasidion* hosts. It can be debilitating or beneficial, or sometimes there is no significant change. The growth rate experiments also showed that infection by multiple viruses does not mean more debilitating effects on the growth rate.

In conclusion, it is possible to infect *Heterobasidion* isolates with multiple partitiviruses to generate new virus-host combinations to be tested as putative biocontrol strains. However, more experiments need to be done regarding more virus compositions and more recipients. In the future, it will be interesting to compare the influence of single virus and multiple virus infections.

Avainsanat — Nyckelord — Keywords

Heterobasidion annosum sensu lato, *Heterobasidion parviporum*, mycovirus, multi-partitivirus transmission, new host, biocontrol

Säilytyspaikka — Förvaringsställe — Where deposited

Master's Program in Agricultural Sciences, Department of Agricultural Sciences

Muita tietoja — Övriga uppgifter — Further information

Supervisors: Prof. Jarkko Hantula (Luke), Eeva Vainio (Luke)

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List of abbreviations

aka = also known as

bp = base pair

CThTV = *Curvularia* thermal tolerance virus

CP = coat protein

DEPC= Diethyl pyrocarbonate

DNA = double strand nucleic acid

ds = double stranded

H. = *Heterobasidion*

HetRV6 = *Heterobasidion* RNA virus 6

HetPV = *Heterobasidion* partitivirus

IGPs = Intersterility groups

ICTV = International Committee on Taxonomy of Viruses

RdRP = RNA dependent RNA polymerase

RNA = ribonucleic acid

RT = reverse transcription

s.l. = *sensu lato*

sp. = Species, especially when the genus is known, but the species is uncertain or unspecified. (plural: "spp.")

spp. = Species pluralis

ss = single stranded

stdev = standard deviation

var.= variety

1 Introduction

1.1 *Heterobasidion* fungus overview

1.1.1 Introduction and history

Heterobasidion spp. is a forestry fungus that grows on conifers and causes root and butt rot. It belongs to the phylum of *Basidiomycota* and the family of *Bondarzewiaceae* (Hibbett & Donoghue 1995). It is a very common and widespread forest pathogen in northern hemisphere (Korhonen et al. 1998a). *Heterobasidion* is economically the most important disease of conifers in the north temperate regions, in Europe alone, the economic loss is reckoned around 800 million euros per year (Asiegbu et al. 2005). According to Hellgren and Stenlid (1995), the loss caused by reduction in diameter growth of infected trees could add a further 70% to the direct economic loss caused by decay.

In field, the fruit bodies of *Heterobasidion* are found mostly on stumps or dead trees, sizes vary between 1 cm to 40 cm across, the top surface is often reddish or dark brown and the lower surface shows white when it is still actively growing and starts to become cream-colored as it ages (Greig 1998).

The first one who described the fungus was Fries 1821 referred in Asiegbu et al. 2005, he described the fruit body of the fungus on spruce and named it *Polyporus annosum* (Fr.), but he did not relate it to any tree disease. German forestry scientist Robert Hartig 1874 referred in Asiegbu et al. 2005 first described the correct fruit body of *Heterobasidion annosum* and first related it to the conifer disease in his book *Important Diseases of Forest Trees*. Before him, many scientists think fungus has nothing to do with tree diseases (Hüttermann & Woodward 1998). Despite his important work and influence on forest pathology, Robert Hartig did not make any progress in the taxonomy of *Heterobasidion annosum* because he placed the fungus into a wrong genus which he named *Trametes* (Hüttermann & Woodward 1998).

The genus name *Heterobasidion* was established by Oscar Brefeld in 1888, he inherited the species name *annosum* from Fries and since then, the full correct name for this fungus is *Heterobasidion annosum* (Hüttermann & Woodward 1998). The meaning of *H. annosum* has changed thereafter into a species cluster. Brefeld is also the first scientist who cultivated the complete life cycle of *H. annosum* in the laboratory in 1888 referred in Hüttermann & Woodward 1998.

1.1.2 Taxonomy

Heterobasidion genus is consisted of many species. Recent studies about *Heterobasidion* have revealed more species. There are mainly two big species cluster: *H. annosum* and *H. insulare*.

H. annosum species complex was considered as a single species until mating experiments proved the existence of intersterility groups (ISGs) (Korhonen 1978). When talked as a species cluster, it is often referred to as *H. annosum sensu lato* (s.l.). *H. annosum* s.l. is composed of three European species *H. annosum sensu stricto*, *H. parviporum* Niemelä & Korhonen and *H. abietinum* Niemelä & Korhonen, two North American species *H. irregulare* and *H. occidentale* (Niemelä & Korhonen 1998, Otrosina & Garbelotto 2010).

H. insulare was found to be species complex too (Dai et al. 2002). *H. insulare* has been split into many species: *H. amyloideum* Y. C. Dai et al., *H. australe* Y. C. Dai & Korhonen, *H. ecrustosum* Tokuda et al., *H. linzhiense* Y. C. Dai & Korhonen, *H. orientale* Tokuda et al., *H. tibeticum* Y. C. Dai et al. (Ota et al. 2006; Dai et al. 2007; Dai & Korhonen 2009; Tokuda et al. 2009; Chen et al. 2014). Recently Zhao et al. (2017) found a new species *H. amyloideopsis*, it formed a monophyletic group within the *H. insulare* complex as a sister to *H. amyloideum*.

Another species, *H. araucariae* P. K. Buchanan—is classified outside of the two complexes mentioned above, and found in Australia, New Zealand and adjacent regions (Buchanan 1988).

1.1.3 Infection biology and disease symptoms

Most *Heterobasidion* species are saprotrophs (Dai & Korhonen 2009). *H. annosum* s.l. complex is capable of acting as necrotrophs on living trees but also saprotrophs when decomposing already dead wood. *H. annosum* s.l. is generally acknowledged the most destructive pathogen to conifer forests (Asiegbu et al. 2005). Species of the *H. insulare* species complex are considered mostly a harmless non-pathogenic saprotroph. However, *H. insulare* has been reported by Yen et al. (2002) to cause root and butt rot on *Pinus luchuensis* in Taiwan.

Heterobasidion can produce both conidiospores and basidiospores. Conidiospores are vegetative (asexual) spores and basidiospores are sexual spores developing in fruiting bodies. Basidiospores play the main role in its infection biology by landing on stump surfaces or wounds of roots and stems (Redfern and Stenlid 1998). Basidiospores are released in summer in temperate regions because summer is the most susceptible season for trees stumps (Redfern and Stenlid 1998). It is also the best season for the fungus to grow, as the optimum temperature of *Heterobasidion* growth is at 22-28 °C (Korhonen & Stenlid 1998). When the temperature drops below 5 °C, it is much less frequently for the fungus to establish (Meredith 1959). Over 35 °C, the fungus is not able to infect trees (Ross 1973). Once established on the stumps, the spores start to grow very fast and soon the inner part of the stump and the roots are infected too (primary infection). Then small white pads of mycelium start to form on the stumps or injured roots and this is the first stage of formation of its fruit body (Greig 1998). Basidiospores can only infect injured roots and stumps, although they can possibly penetrate the tree bark to a small extent, they are not able to infect healthy uninjured roots (Peek et al. 1972). However, secondary infection can take place when the roots meet each other underground (Redfern & Stenlid 1998). In the primary infection, single basidiospore produces homokaryotic mycelium; in the secondary infection, the mycelium is a mosaic of homokaryotic and heterokaryotic mycelia (Hansen et al. 1993).

In field, *H. annosum* generates layers of white, paper-thin mycelium beneath the bark of the infected roots, but this is not a diagnostic feature because other root rot pathogens

produce similar mycelium too, so it is not possible to tell them apart in field condition (Greig 1998). Also, the symptoms of *H. annosum* on living trees are not characteristic, sometimes the infection even causes no external symptoms in spruce as it develops within the stem of a living tree (Greig 1998).

The symptoms of *H. annosum* may vary on different ages of coniferous trees, on young trees, the disease causes the needles turn red first and then brown, eventually kills the young trees within one season; on older trees however, decline of annual shoots may last ten years before the tree dies (Greig 1998). *H. parviporum* only rarely kills spruces, but trees with rotten roots or stems tend to break down in strong wind (Niemelä et al. 2005). *Heterobasidion* fungus may exist in the dead stumps and roots for decades, remaining from one rotation to the next, therefore it is often called "diseases of the site" (Greig & Pratt 1976).

1.1.4 Distribution and host range

Based on previous studies, *Heterobasidion* is widely distributed in the Northern hemisphere, including Europe, North America, Russia, China, and Japan (Garbelotto & Gonthier 2013). The genus *Heterobasidion* has a host range of more than 200 plant taxa (Korhonen & Stenlid 1998).

H. annosum sensu stricto (s.s.), *H. abietinum* Niemelä & Korhonen and *H. parviporum* Niemelä & Korhonen inhabit in Eurasia (Niemelä & Korhonen 1998). *H. irregulare* Garbel. & Otrosina and *H. occidentale* Otrosina & Garbel are found in North America (Otrosina & Garbelotto 2010).

H. annosum s.s. grows on pines (Pinus spp.), especially Scots pine (*Pinus sylvestris L.*), it is found all over Europe except the northernmost area, and its distribution regions extend east to southern Siberia (Dai et al. 2006). *H. parviporum* mostly attacks Norway spruce [*Picea abies* (L.) Karst.], the distribution is mainly in northern Europe but it is also found in central and south Europe, China, Japan and Sothern Siberia (Dai et al. 2003; Tokuda et al. 2011); *H. abietinum* infects species of silver fir (*Abies*), it is mainly

distributed in Mediterranean countries; *H. araucariae* Buchanan inhabits dead wood of *Araucaria*, *Agathis* and *Pinus*, it has been reported from eastern Australia, New Zealand, Papua New Guinea and the Fiji Islands; *H. insulare* as a species complex is a harmless saprophyte that grows on dead wood of *Abies*, *Pinus* and *Picea*, it is found in southern and eastern Asia including China, Russia and Japan (Niemelä & Korhonen 1998).

Both *H. irregulare* and *H. occidentale* inhabited in North America, until during World War II, *H. irregulare* was brought into central Italy (Gonthier et al. 2004), since then it has been invasively spreading in Italian stone pines (*Pinus pinea* L.) (Gonthier et al. 2007). *H. irregulare* lives on pines, junipers and incense cedars whereas *H. occidentale* has a broader host range within *Abies*, *Picea*, *Tsuga*, *Pseudotsuga* and *Sequoiadendron* (Garbelotto & Gonthier 2013).

1.2 Disease control against *Heterobasidion*

Scientists have been searching control methods against *Heterobasidion* for a long time. In early stage of study, even some great scientists made mistakes on how to control the disease. For example, Robert Hartig proposed digging sanitary ditches around the tree and later in his article labeling this method as the most important means when it comes to controlling of *H. annosum* (Hüttermann & Woodward 1998). His method totally neglected the dispersal of fungus spores. The injury of roots was unavoidable because of the digging and cutting, thus creating more entries for the fungus and increasing the fungus infection. Currently, *H. annosum* s.l. is one of the few forest pathogens that can be and has been controlled in managed forests (Garbelotto& Gonthier 2013). Although it is not possible to eliminate the fungus completely after it has been established on the tree stump, it is possible to find control methods against this disease by using knowledge of its host preference and its infection biology.

1.2.1 Silvicultural control

By taking advantage of the knowledge of the biology and interactions of the fungus, hosts and the environment, silvicultural control uses forest management methods to reduce harm caused by the disease.

It has been proved that *Heterobasidion* root and butt rot can be controlled through stump removal and the removal of all roots (Cleary 2013), so far this is also one of the only two methods that can make a difference in heavily infected forests (Gibbs et al. 2002). In order to be 100% effective, stump removal has to be performed strictly for the reason that *Heterobasidion* fungus is able to survive and transfer to consecutive stand even in 1-cm-thick roots (Greig 1984). In a recreation site in South California, all infected trees and at least one row of healthy trees were uprooted, also a 150 cm deep trench was dig up, the method was proved effective and the cost was not considered excessively high (Kliejunas et al. 2005). Nevertheless, this method is likely to make the injured roots of the healthy trees more susceptible to airborne infections (Korhonen et al. 1998b). The use of machines when conducting stump removal is also inapplicable in most forest sites. In addition, some negative impact to the environment cannot be neglected (Walmsley & Godbold 2010). Therefore, stump removal is not used frequently in practice.

Another method is to control the timing of logging and thinning, focuses more on preventing and limiting the airborne infections. To lower the risk of infection during logging, it is crucial to carry out logging in the right season, when temperature does not favor the spore germination. In Northern Europe, logging is conducted in winter below 0 °C (Brandtberg et al. 1996), whereas in southeastern USA, it is applied in summer when the temperature is over 40 °C (Ross 1973).

An additional silvicultural management is simply by planting a tree species not susceptible or less susceptible to *Heterobasidion*. For example, planting deciduous species is an option because they are relatively less susceptible to *Heterobasidion* than conifers (Delatour et al. 1998). It is reported that the loss caused by *Heterobasidion* in pure stands is more than that in mixed stands (Linden & Vollbrecht 2002), so planting a mixed species of trees is also feasible in terms of controlling the disease. The change of tree species is the other method that can make a difference in heavily infected forests. If

the forest site is kept free of conifers for a rotation cycle for more than 50 years, most of *Heterobasidion* dies out with the decaying roots during that time.

1.2.2 Chemical control

Among the trials of many chemicals, urea and borates are proved effective and the most suitable to be used in the forests, as they are easily obtainable, simply to handle, inexpensive and non-toxic (Pratt et al. 1998).

Rishbeth (1959) conducted the first chemical stump treatment using 20% aqueous urea, and it was reported to reduce the growth of inoculated *H. annosum* on *Pinus sylvestris* discs. Since then, a number of tests have been done across Europe and North America, these trials reveal that urea at the concentration over 20% can provide sufficient control on pine stumps (Pratt et al. 1998). In Finland urea solutions over 30% are used in practice (Johansson et al. 2002, Nicolotti & Gonthier 2005). When urea is applied on wood tissues, urease, which is generated by the wood, catalyzes the hydrolysis of urea (Johansson et al. 2002). Hydrolysis of urea produces ammonium ions, which leads to a rapid increase of pH (Pratt et al. 1998). The germination of *H. annosum* spores takes place at pH 3-7.5, with the optimum pH between 4 and 6.5 (Schwantes et al. 1976 referred in Pratt et al. 1998). *H. annosum* is able to grow in a wide range of pH, but the optimum pH for growth is between 4 and 5.7, depending on the strain (Korhonen & Stenlid, 1998). So, when the pH rises over the level at which the spores can survive, and the level at which the mycelium can grow, the germination and growth of *H. annosum* is prevented (Johansson et al. 2002). Though worked well on pine, the effect of urea is not as good on spruce and other genera (Pratt et al. 1998).

The primary mode of action of borates is based on the metabolism of basidiomycetes (Lloyd 1997). Rishbeth (1959) discovered that although boron was consumed up by above-ground pine stumps after two months of treatment, its fast penetration can still provide toxic barrier to colonization of basidiospores.

The downside of chemical treatment is its environmental issues. Both urea and borates can lead to serious damages to general ground-vegetation species (Westlund & Nohrsted 2000). Urea is toxic to plants at high concentration (Krogmeier et al. 1989). Another concern to the environment is that the release of a sufficient quantity of urea and borates can result in the contamination of water (Pratt et al. 1998). As a result, the forestry practice in European Union and UK targets on minimizing the use of chemical pesticides in forests (DIRECTIVE, H. A. T. 1994, UKWAS 2000, Willoughby et al. 2004).

1.2.3 Biocontrol methods

The ecological relationship between one organism and other organisms is often antagonistic (Campbell 1989). This gives researchers the idea of using such microorganisms to control *H. annosum*. The advantage of biocontrol is clear, first, the biocontrol agents are biodegradable, which is good to the environment; second, the effect of biocontrol is likely to be sustainable because the biocontrol agent might become a consistent part of the biocoenosis (Andrews 1990).

A large number of different fungi have been tested for antagonistic activity against *H. annosum*, among them, *Phlebiopsis gigantea* (Fr.) Jülich has been reported to be the most effective one (Holdenrieder & Greig 1998). Studies in England show that inoculation of *P. gigantea* can decrease the existence of *H. annosum* root rot significantly (Rishbeth 1963, Tubby et al. 2008). In United States, Hodges (1964) found the same effect of *P. gigantea*. Currently, there are three products of *P. gigantea* in Europe: PG Suspension from UK, PG IBL from Poland and Rotstop in Finland (Korhonen et al. 1994). One concern of the biocontrol method using *P. gigantea* is its efficiency, it was indicated that the efficiency of the product depends on concentration of the suspension applied on tree stumps and the growth rate of *P. gigantea* (Sun et al. 2009). Another issue is that *P. gigantea* is not used universally, in USA *P. gigantea* product named Rotstop C is registered by the Environmental Protection Agency (EPA Registration Number: 64137-12) in some states: Alabama, Florida, Georgia, Michigan, North Carolina, South Carolina, Virginia and Wisconsin (US Rotstop FAQ 2017). The

approval of other states' registrations is still pending; in Italy, none of the three formulations of *P. gigantea* is registered due to the restriction on introduction of extraneous organisms (Annesi et al. 2005). Apart from all the methods mentioned above, there is a need for new ways to control and manage *Heterobasidion* spp.

1.3 Mycovirus

Viruses are extremely small transmittable life forms that reproduce by infecting living cells (Guttman 2013). Viruses that infect fungi are called mycoviruses (Ghabrial & Suzuki 2009). Although discovered relatively recently, mycoviruses are considered to be of ancient origins (Ghabrial et al. 2015).

Mycoviruses were first reported definitively in 1962, when they were found to infect cultivated button mushroom *Agaricus bisporus* (Hollings 1962). Studies show that mycoviruses occur in all major groups of fungi and the result of random sampling of fungus cultures reveals that 10 to 15% of fungus species carry mycoviruses (Bozarth 1972). Further studies indicate that prevalence of mycoviruses among phytopathogenic fungi is more than 80% (Ghabrial & Suzuki 2009).

Although there are findings showing that ssDNA mycovirus is infectious as a free particle (Yu et al. 2013), most mycoviruses are considered lack of extracellular phase and transmitted intracellularly during cell division, sporogenesis or cell-to-cell fusion (hyphal anastomosis) (Ghabrial et al. 2015).

1.3.1 Mycovirus taxonomy

There are three types of genome composition of mycoviruses: double-stranded RNA (dsRNA), single-stranded RNA (ssRNA) and very rarely DNA (Yu et al. 2010). Fungal viruses with linear dsRNA genomes are classified into seven families: *Chrysoviridae*, *Endornaviridae*, *Megabirnaviridae*, *Quadriviridae*, *Partitiviridae*, *Reoviridae* and

Totiviridae; fungal viruses with genomes composed of linear positive-sense ssRNA are classified into five families: *Alphaflexiviridae*, *Barnaviridae*, *Gammaflexiviridae*, *Hypoviridae* and *Narnaviridae*; fungal viruses with genomes composed of linear negative-sense ssRNA are classified into proposed family *Mymonaviridae*; at last, fungal viruses with circular ssDNA are classified into new family *Genomoviridae* (Ghabrial et al. 2015, Varsani & Krupovic 2017, Amarasinghe et al. 2017). The only mycovirus with ssDNA genome discovered is a hypovirus from the phytopathogenic fungus *Sclerotinia sclerotiorum* (Yu et al. 2010). Scientists have yet to find fungal viruses with dsDNA genomes, however, it is possible that they exist because dsDNA viruses were found in water mold *Rhiziodiomyces* sp. which is considered as protista not fungi (Dawe and Kuhn 1983). In this thesis, *partitiviridae* is the family focused and studied.

1.3.2 Mycovirus effects

Although most mycoviruses do not cause obvious symptoms on their fungus hosts (Ihrmark 2001; Ghabrial & Suzuki 2009), the symptoms of mycoviruses infection is not always latent, but can be diverse.

Some mycoviruses can mediate hypovirulence in their fungal hosts, thus reducing or even depriving the virulence of fungal pathogens (Nuss 2005). Therefore, mycoviruses can be exploited as biocontrol agents against their phytopathogenic fungal hosts in Europe (MacDonald & Fulbright 1991). The most successful case is the utilization of hypovirus against *Cryphonectria parasitica*, a phytopathogen that causes chestnut blight in Europe (Anagnostakis 1982). An *Endornavirus* from the violet root rot fungus, *Helicobasidium mompa,* is a hypovirulence factor to its host (Osaki at al. 2006). There are many examples of partitiviruses conferring hypovirulence too. For example, *Rhizoctonia solani* partitivirus 2 confers hypovirulence in the phytopathogenic fungus *Rhizoctonia solani* (Zheng et al. 2014.), Sclerotinia sclerotiorum partitivirus 1 conferred hypovirulence on its natural host *Sclerotinia sclerotiorum* strain WF-1, a phytopathogen that causes white mold (Xiao et al. 2014).

There are other examples of using mycoviruses against fungal diseases except for their hypovirulence. A *megabirnavirus* named *Rosellinia necatrix megabirnavirus* 1 (RnMBV1) from the white root rot fungus *Rosellinia necatrix* has the potential for biological control (Chiba et al. 2009). *Botrytis porri* RNA virus 1 (BpRV1) from the family *Botybirnavirus* can reduce the virulence of its host *Botrytis porri*, a pathogenic fungus that causes garlic clove rot, garlic leaf blight and leek leaf rot (Wu et al. 2012). *Aspergillus fumigatus* partitivirus 1 resulted in significant aberrant phenotypic alterations and attenuation of growth of the fungus host *Aspergillus fumigatus,* which causes human disease with an immunodeficiency (Bhatti et al. 2011). All these successful exploitations of mycoviruses give scientists hope to find more fungal viruses to control fungal diseases. As the most severe conifer pathogen, *Heterobasidion* receives its attention too.

Some mycoviruses can enhance the virulence of their hosts. *Talaromyces marneffei* partitivirus 1 increased the virulence of *Talaromyces marneffei* in mice, causing shorter survival time and higher fungal burden in their organs (Lau et al. 2018).

Some mycoviruses can form a mutualistic association with their hosts. For example, *Curvularia* thermal tolerance virus (CThTV) from endophytic fungus *Curvularia protuberate* is involved in a three-way symbiosis with both its host fungus and a tropical panic grass, providing the host fungus and the grass thermal tolerance at high soil temperature in Yellowstone national park (Marquez et al. 2007).

The effects of mycoviruses infection is not always immutable. It was reported that the effects of *Heterobasidion* viruses on the host fungi is dependent on the temperature (Vainio et al. 2010, 2012). Further studies show that a single virus strain causes different effects on different *Heterobasidion* hosts and that under different environmental and ecological conditions a single virus strain can mediate beneficial, cryptic or detrimental effects on a single host (Hyder et al. 2013).

The research of exploiting partitiviruses to control *Heterobasidion* is still rather new and ongoing. However, based on researches done before (Ihrmark et al. 2004, Vainio et al. 2010, Hyder et al. 2013, Vainio et al. 2018b), there are already some great

breakthroughs and the future is quite promising. But previous studies focus on single species partitivirus biocontrol, the exploration of multi-partitiviruses is still limited.

1.4 *Heterobasidion* partitivirus

1.4.1 Partitivirus general information

Partitiviridae is a family of small, isometric, non-enveloped dsRNA viruses with bisegmented genomes from 3kbp to 4.8kbp (Vainio et al. 2018a). They are named *Partitiviridae* because they have bisegmented genomes. The two genomes encode for CP and RdRP respectively (Bozarth et al. 1971) and they are separately encapsidated (Vainio et al. 2018a). Normally the larger segment (dsRNA1) encodes for RdRP and the smaller one (dsRNA2) encodes for CP (Ghabrial et al. 2015).

According to the newest report from the International Committee on Virus Taxonomy (ICTV) in 2018, the family *Partitiviridae* consists of five genera: Alphapartitivirus, Betapartitivirus, Gammapartitivirus, Deltapartitivirus and Cryspovirus. Each one has its own characteristic hosts: Alphapartitivirus and Betapartitivirus infect either plants or fungi, Gammapartitivirus grows on fungi, Deltapartitivirus is found on plants and Cryspovirus is found on protozoa (Vainio et al. 2018a).

In this thesis, I focus only partitiviruses infecting fungi. Same as most other mycoviruses, partitiviruses cannot form extracellular particles and the transmission of partitiviruses relies on cell division, spores or hyphal cell fusion between genetically compatible fungal strains (Nuss 2011), but also between incompatible or even intersterile strains (Ihrmark et al. 2002, Vainio et al. 2017).

1.4.2 Partitiviruses found in *Heterobasidion*

According to Vainio et al. (2018a), viruses of families *Partitiviridae, Narnaviridae* and the unassigned *Heterobasidion* RNA virus 6 (HetRV6) are all found on *Heterobasidion* spp. It was reported that around 15-17% *Heterobasidion* l. isolates host dsRNA viruses, among them, almost one third is partitiviruses (Ihrmark et al. 2001). *Heterobasidion* spp. hosts a variety of partitiviruses (Ihrmark 2001). According to Nibert et al. (2014) and Kashif et al. (2015), HetPV1, HetPV3, HetPV4, HetPV5, HetPV9, HetPV12, HetPV13, HetPV14 and HetPV15 are classified into family Alphapartitivirus. HetPV-P, HetPV2, HetPV7, HetPV8 are classified into family Betapartitivirus (Nibert et al. 2014). Recent study shows HetPV16 and HetPV20 should be included into family Alphapartitivirus too (Hyder et al. 2018).

Another important finding is that *Heterobasidion* partitiviruses rarely have an impact on the growth of their natural hosts but can affect the growth of their new host more frequently (Jurvansuu et al 2014), which shows us its potential as a biocontrol agent against *Heterobasidion* spp.

1.4.3 Transmission of *Heterobasidion* partitiviruses

It has been shown in many studies that *Heterobasidion* partitiviruses are able to pass on from one *Heterobasidion* species to another (Ihrmark et al. 2002, Vainio et al. 2010, Vainio et al. 2012, Hyder et al. 2013, Jurvansuu et al. 2014, Vainio et al. 2017).

In their host fungus, *Heterobasidion* partitiviruses reproduce inside the cytoplasma (Vainio et al. 2015) and they do not leave the cytoplasm of their hosts (Ghabrial 1998). They can spread vertically via both asexual conidiospores and sexual basidiospores (Ihrmark et al. 2002, Ihrmark et al. 2004). As they lack extracellular particles, like other mycoviruses, their transmission between fungal strains mainly occur by anastomosis (Vainio et al. 2015). Studies from Ihrmark et al. (2002) indicates that dsRNA viruses can be transmitted between incompatible *Heterobasidion* isolates via anastomosis in the laboratory.

Vainio et al. (2017) showed that it is easy for partitiviruses of *Heterobasidion* to be transmitted both in the laboratory and in nature between incompatible strains and even distantly related *Heterobasidion* species.

1.4.4 Diverse phenotypic effects of *Heterobasidion* partitivirus infection

Heterobasidion partitiviruses are, like other mycoviruses, mostly cryptic (Ihrmark 2001), however, this is not always the case. It has been reported that the germination frequency of basidiospores from infected *Heterobasidion* were reduced due to partitivirus infection (Ihrmark et al. 2004). According to Vainio et al. (2010), at low temperature, HetRV3-ec1 originally from *H. ecrustosum* shows a negative effect on the growth rate of *H. abietinum*, but this phenomenon does not apply to *H. irregulare* and *H. ecrustosum*, or to *H. abietinum* at higher temperature. The most common virus infecting *H. annosum* s.l. is *Heterobasidion* RNA virus 6 (HetRV6). It is not a partitivirus and it has not been assigned into any family yet, surprisingly it has a slightly positive effect on the growth rate of *H. annosum* and *H. parviporum* (Vainio et al. 2012). Vainio et al. (2012) also indicated that HetRV6 accounts for around 70% of dsRNA infections in European *Heterobasidion* strains. HetRV6 is believed to be taxonomically close to *Curvularia* thermal tolerance virus (CThTV) because they share a similar polymerase (Marquez et al. 2007).

Further studies from Hyder et al. (2013) show that the effect of *Heterobasidion* partitiviruses can be cryptic, beneficial and detrimental under different environmental or ecological conditions. Recent study from Vainio et al. (2018b) showed that HetPV13-an1 has a major negative effect on the growth of its host.

All these researches indicate that *Heterobasidion* partitiviruses have a diverse effect on their hosts, which shows us a potential new way to control *Heterobasidion* spp.

1.4.5 Multiple virus co-infection

Simultaneous infection of a single fungal strain by two or more virus particles is called co-infection. According to Ghabrial (1998), co-infection is common among mycoviruses, he also believed that co-infection is the consequence of how mycoviruses are transmitted in nature. Ikeda et al. (2005) analyzed 83 isolates of the violet root rot fungus *Helicobasidium mompa* for five years and found dsRNA viruses accumulated over time. Park et al. (2005) reported that co-infection of two distinct mycoviruses (CeRV1 and CeRV2) exists in *Chalara elegans*, a plant pathogenic fungus that causes black root rot disease, which is the first time that co-infection was found in *Chalara elegans*. In white root rot fungus *Rosellinia necatrix*, novel unknown dsRNA viruses were noticed to appear after 2-3 years in an apple orchard (Yaegashi et al. 2012). Wu et al. (2016) found that plant pathogenic fungus *Sclerotinia nivalis* was co-infected by three different mycoviruses. Co-infection also occurs in alkalophilic fungus *Sodiomyces alkalinus*, it was demonstrated to be infected by three dsRNA viruses, a novel betapartitivirus, a gammapartitivirus and a novel fusarivirus (Hrabakova et al. 2017). Tuomivirta and Hantula (2005) found out that a single isolate of *Gremmeniella abietina* var. *abietina* type A, which causes conifer cankers in Europe and North America, hosted three unrelated viruses, named respectively *Gremmeniella abietina* mitochondrial RNA virus S2 (GaMRV-S2), *Gremmeniella abietina* RNA virus MS2 (GaRV-MS2) and *Gremmeniella abietina* RNA virus L2 (GaRV-L2). Recently Botella and Hantula (2018) described that *Gremmeniella abietina* hosts a distinct and plentiful community of viruses of different genera, furthermore, co-infection by multiple virus may affect the phenotype of the host.

Co-infection causes various phenotypic effects on fungal hosts. Ikeda et al. (2005) did not find any correlation between virulence level of fungal host and the presence of specific dsRNAs. In Wu's study, co-infection had no obvious effects on the fungal host *S. nivalis* (Wu et al. 2016). In Hrabakova's study, no apparent phenotypic alteration to the fungal host was found either (Hrabakova et al. 2017). Sometimes, however, co-infection can cause hypovirulence to fungal hosts. *Rosellinia necatrix* megabirnavirus 2 (RnMBV2) was able to confer hypovirulence together with *Rosellinia*

necatrix partitivirus 1 (RnPV1) on host fungus *Rosellinia necatrix*, however on its own, neither was able to cause hypovirulence (Sasaki et al. 2016).

Co-infection is also common among *Heterobasidion* spp. More studies have demonstrated that multiple virus infection does happen in *Heterobasidion* fungus (Vainio et al. 2012, 2013, 2015, 2016, Kashif et al. 2015, Hyder et al. 2018). Interestingly, in one study it was even found that two mycoviruses (HetRV4 and HetRV6) were able to survive after their host fungus *H. parviporum* was digested by insect *Hylobius abietis* feeding on wood of *Pinus sylvestris* branches inoculated by *H. parviporum* (Drenkhan et al. 2013). In my thesis, I tested whether multiple virus co-infection would show features making them useful for biocontrol against *Heterobasidion* species.

2 Research objectives

The main objective of this thesis is to find out whether it is possible for multiple partitiviruses to be transmitted together from one *Heterobasidion* host to another, and to understand the phenotypic effects of multiple partitiviruses on the growth rate of the new hosts. Furthermore, this thesis aims to compare the infection effects from different combinations of multi-partitiviruses on the same fungal host. At last, this thesis is trying to find new biocontrol methods using partitiviruses co-infection and further understanding the relation of multi-partitiviruses and their *Heterobasidion* hosts.

In this thesis, the main hypothesis is that the phenotypic effects of different combination multi-partitiviruses on the same *Heterobasidion* host will be different, and that the more viruses exist in the host, the more debilitating effects occur.

3 Materials and methods

3.1 Materials

3.1.1 Fungus isolates and viruses used in this thesis

All the fungal isolates in this study were provided by Eeva Vainio and Rafiqul Hyder from Natural Resources Institute Finland (Luke). The origins information about all the isolates used in this thesis can be found in Table 1. The fungal strains used in this study can be divided into two groups. One group of fungi contains multiple viruses and is called donor group. The other group of fungi is completely virus free, is called recipient group. The experiment is designed to measure, whether multiple viruses can be transmitted from the donor strains to the recipient strains. There are two isolates of *Heterobasidion* fungus in the donor group, both of them are *H. parviporum*: *H. parviporum* LAP3.2.2*5 and *H. parviporum* 7R242*5, here they are named short for MV1 and MV2 respectively, "*5" means hosting five viruses. The full name of the donors was combined by two parts, the first part is the name of the host fungus, and the other part is the summary name of the five hosting viruses. Each donor isolate contains five different viruses, *H. parviporum* LAP3.2.2*5 carries HetPV2, HetPV4, HetRV6, HetPV13 and HetPV16; *H. parviporum* 7R242*5 contains HetPV2, HetRV6, HetPV7, HetPV9 and HetPV13. The information on the donor group is concluded in Table 2.

There are ten different isolates of *Heterobasidion* fungus in the recipient group, they are all virus free *Heterobasidion* isolates, six of them belong to *H. annosum*, and are named S49-5, 03021, HA5.31, T60-9, 93173, RKON1.60; the other four isolates belong to *H. parviporum*, and are named RK5A, 7R18-11, SB10.16, EV0789. The information of the recipient group is concluded in Table 3.

Table 1 Origin of *Heterobasidion* fungal isolates. * Not an original fungal strain.

Isolate	Collected from	Year	Collector
H. parviporum (LAP 3.3.2)	Lapinjärvi	1991	T. Piri
H. parviporum (7R242)	Ruotsinkylä, Tuusula	2005	T. Piri
H. annosum (S49-5*)	Läyliäinen, Loppi	2005	T.Piri & H.Nuorteva
H. annosum (03021)	Kopparö, Tammisaari	2003	Kari Korhonen
H. annosum (HA5.31)	Harju	2007	Tuula Piri
H. annosum (T60-9)	Läyliäinen, Loppi	2005	T.Piri & H.Nuorteva
H. annosum (93173)	Liljendal	1993	Kari Korhonen
H. annosum (RKON1.60)	Ruotsinkylä, Tuusula	1993	Tuula Piri
H. parviporum (RK5A)	Tuusula	2010	E. Vainio, T. Piri
H. parviporum (7R18-11)	Ruotsinkylä, Tuusula	2005	Tuula. Piri
H. parviporum (SB10.16)	Solböle, Raasepori	2005	Tuula Piri
H. parviporum (EV0789)	Evo, Hämeenlinna	2008	Tuula Piri

Table 2 Donor fungus strains. *5 means that the fungus hosts five viruses.

	Short name	Full name	Original strain before virus transmissions	Viruses hosted
Donor fungus strains	MV1	LAP3.2.2*5	*H. parviporum* LAP3.2.2	HetPV2, HetPV4, HetRV6, HetPV13 and HetPV16
	MV2	7R242*5	*H. parviporum* 7R242	HetPV2, HetRV6, HetPV7, HetPV9, HetPV13

Table 3 Recipient fungus strains.

Recipient fungal strains	
H. annosum	*H. parviporum*
S49-5	RK5A
03021	7R18-11
HA5.31	SB10.16
T60-9	EV0789
93173	
RKON1.60	

3.1.2 Growth media

Malt extract agar plates (2%) and modified orange serum (MOS) agar (Müller et al. 1994) were used as growth medium (Appendix 1) in this thesis. The transmission experiment and the pairing test experiment were done on malt plates.

MOS (+) plates were used to grow fungus and to sever the agar and the fungus mycelium, so that the fungus mycelium could be collected for RNA extraction later, "+" in bracket after “MOS” means the MOS medium was layered with a piece of special autoclaved cellophane membrane (Visella OY) which separates the medium and the mycelium. The membranes are the same shape and size with the medium plates.

3.2 Methods

3.2.1 Transmission experiments

Transmission experiment was conducted as described by Ihrmark et al. (2002). The virus was transmitted from donor to recipient fungal strain via anastomosis. Each donor is paired with all ten recipients, which makes it 20 pairings in total because there are two donors. To acquire more accurate results, each pairing is replicated, which makes

40 plates totally. The purpose of transmission experiment was to allow the virus transmission to happen and produce 20 different fungal strains infected by as many viruses as possible, and later the fungal strains successfully transmitted by multiple viruses (at least three) can be used in further experiments to find out how different combinations of multiple virus infection affect the host fungus.

Fungal isolates of the donor and the recipient fungal strain were placed on opposite sides on the petri dish as is shown in Figure 1. Donor strain on the left contained five different viruses. Recipient strain on the right was virus free before transmission experiment started. A hyphal barrier clearly formed in the middle between the two isolates (Figure 1). The plates were incubated for four weeks at 20 °C until the two fungal strains formed hyphal contact. And then the viruses can be transmitted through anastomosis. In the next step three small samples were taken from the recipient's side and later cultured on Malt plate for further experiment, far from the demarcation line (Figure 1).

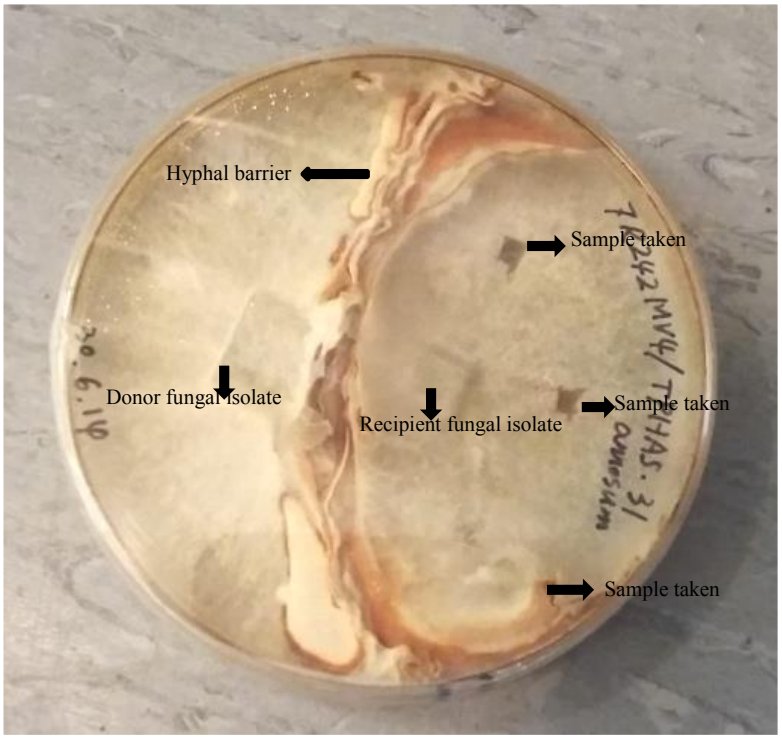

Figure 1 Transmission experiment on a petri dish between two *Heterobasidion* fungal isolates. Donor strain on the left contains five different viruses. Recipient strain on the right is virus free. A hyphal barrier clearly forms in the middle between the two isolates.

3.2.2 Pure culture of virus infected recipient

After transmission experiment, pure cultures of virus infected recipient fungal strains were established. Three small fungal plugs were taken from the recipient's side, far from the middle demarcation line to avoid mixing with donor fungal cells (Figure 1, 2). Then three cultures were combined on one plate to avoid sampling putatively virus-free sectors of the recipient. The establishment of pure cultures is demonstrated in Figure 2. On the left is the transmission experiment petri dish, on the right is the new malt petri dish where pure culture of infected recipient grows. Orange area is the demarcation line between the donor and the recipient. Red round dot represents the donor and blue round dot represents the recipient. The three small blue squares were the fungus agars picked from the transmission experiment and later transferred to new malt petri dish. Then these fungal plugs were inoculated on 2% Malt extract agar plates and the plates were incubated ten days at 20 °C. There are 40 plates in total. The transfer experiment was done as in Figure 2.

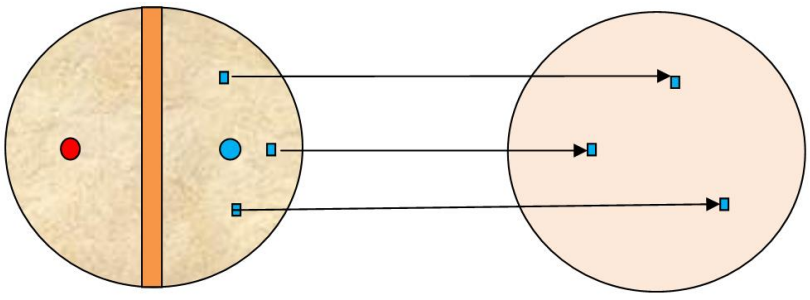

Figure 2 Illustration figure of transfer experiment.

3.2.3 Cell collection of the virus infected recipient fungal isolates

By testing RNA of the recipient mycelium cells, it can be verified whether or not the viruses were successfully transmitted to the recipient. That requires collecting cells from virus infected recipient fungal isolates. The pure culture recipient fungal plugs from 3.2.2 were therefore inoculated onto new modified orange serum (MOS) plates (Figure 3). The 40 plates were incubated for 12 days at 20 °C. In Figure 3, the left plate is the malt plate with pure culture of recipient fungus grows on it. Right plate is new MOS (+) plate. Blue square represents agar containing mycelia. Long arrow means transfer. The blue square in the middle of the left plate was the agar that plugged out and then transferred.

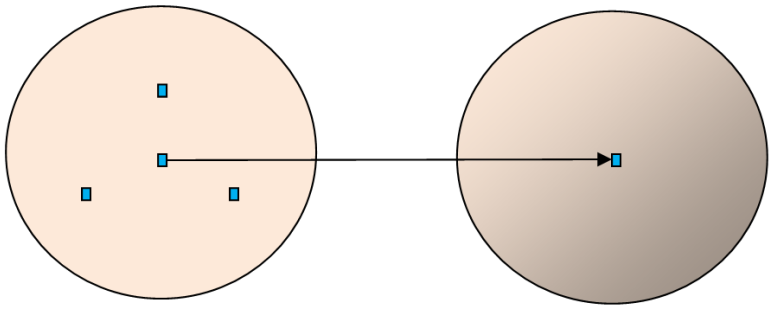

Figure 3 Illustration picture of transferring recipient to MOS (+) plates.

After 12 days growth, mycelium cells on MOS (+) plates were collected into small plastic tubes and marked with the name of the fungus strain, the weight of the mycelium and the date of the experiment. The weight of the tubes was weighed before and after collecting the cells in order to measure the weight of the mycelium cells. The cells' weight was all controlled around 30-50 mg. There are 40 tubes of mycelium cells. These tubes were stored in freezer at the temperature of -20 °C and were used for RNA extraction later.

3.2.4 RNA extraction using Tri Reagent

Heterobasidion partitivirus is an RNA virus, in order to detect its presence, RNA extraction was conducted. Total RNA extraction was carried out using Tri Reagent as the protocol described by Thermo Fisher**.**

As is described in 3.2.3, fresh fungal mycelia were incubated at 20 °C for 12 days on a MOS (+) agar plate. The first step of RNA extraction was the homogenization of fungal mycelia. In this step, 30 to 50 mg fungal hyphae, 0.5-1 cm of sterile quartz sand and 500 ul of Tri Reagent were added into a 2 ml Eppendorf screw cap tube. The cells were broken with FastPrep (4 m/s, 3×20 s) (FastPrep–24™ Tissue and cell homogenizer, MP Biomedicals, USA) and then centrifuged at room temperature at 10000 × g for five minutes. The supernatant layer was transferred to a clean 1.5 ml Eppendorf tube.

The second step of RNA extraction was RNA purification. To purify the nucleic acids, 100 µl of chloroform was added to the supernatant sample. The sample was then mixed with a vortex for 30 seconds and incubated at room temperature for 5 minutes. After incubation the sample was centrifuged at 12000 × g at +4 °C for 10 minutes. The supernatant part was then transferred into a clean 1.5 ml Eppendorf tube. This purification step with 100 µl chloroform was repeated once.

The last step of RNA extraction was RNA precipitation. To precipitate the nucleic acids, 250 µl of isopropanol (the isopropanol bottle was stored in -20 °C freezer) was added into the sample. Then the sample was mixed well with a vortex for 30 seconds and incubated at room temperature for 5 minutes. After incubation the sample was centrifuged at 12000 × g at room temperature for 5 minutes and the supernatant was removed. The pellet left in the tube was washed with 1 ml of ice-cold 75% EtOH and centrifuged at 12000 × g for 5 minutes. The supernatant was removed carefully, and the pellet was either vacuum-dried in a Speedvac for two minutes or put in a laminar hood for 10 minutes to dry. To dissolve the pellet, 50 µl of DEPC-treated sterile water was added to the sample and the sample was placed in room temperature for 15 to 30 minutes. At last, the yield of RNA was determined with spectrophotometer (NanoVue™ spectrophotometer, GE Healthcare, USA). All together there are 40 samples of total RNA from the infected recipient mycelium cells. The details of solutions used in RNA extraction are listed in Appendix 2. The yield of RNA samples is listed in Appendix 3.

3.2.5 Reverse transcription reaction

To obtain complementary DNA (cDNA) of the total RNA we extracted in 3.2.4, reverse transcription reaction was carried out.

First 1 µl of random hexamer primer (dN6, 0.2 µg/ µl) and 12 µl RNA sample were combined into an Eppendorf tube on ice. Then a master mix was prepared on ice. The master mix contains 4 µl of 5 × M–MuLV Revert Aid Reverse Transcriptase Buffer (Thermo Scientific, USA), 2 µl of dNTP mix (10 nM, Solis BioDyne) and 1 µl of Revert Aid M-MuLV Reverse Transcriptase enzyme (200 U/µL, Thermo Scientific, USA) per each sample. Next the samples were denatured in boiling water bath for 5 minutes, then immediately placed on ice. 7 µl of master mix was added into each sample and the reverse transcription started. The samples were centrifuged briefly for about 10 seconds and then incubated at room temperature for 10 minutes. At last the samples were incubated in a Model 2000 Micro hybridization incubator at 42 °C for an hour. The samples were then preserved at -20 °C in a freezer before PCR reaction.

3.2.6 PCR and gel electrophoresis

To detect whether or not the virus in the donor strain was transmitted successfully to the recipient strain, special primers were used in the PCR experiment. Each primer has its own sequence, which can pair with the cDNA we obtained, and each primer has its own annealing temperature. All the primers in this step are listed in Table 4.

Each PCR reaction was conducted in 50 µl volume, including 1 µl of the cDNA template, 1 µl of forward primer and 1 µl of reverse primer (25 µM), 1 µl of dNTP mix (10 mM) (Solis BioDyne, Estonia), 0.25 µl Dream Taq DNA Polymerase enzyme (5 U/µl, Thermo Scientific, USA), 10×Dream Taq Buffer 5 µl (Thermo Scientific, USA) and 40.8 µl of sterile water.

First a master mix was prepared and divided into each tube and then cDNA template was added into each tube. One negative control without cDNA template was added to the PCR too. The PCR reaction was performed with PTC–100® Programmable Thermal controller (MJ Research Inc. USA) by using the following program: first initial denaturation 10 min at 95 °C, followed by denaturation 30 s at 95 °C, annealing time 45 s at temperature specific for each pair of primer, extension 2 min at 72 °C followed by final extension 7 min at 72 °C and cooling at 2 °C. 35 cycles were used for amplification (steps 2 to 4). To check the presence of the virus, PCR products were checked with agarose gel electrophoresis using 1% agarose gel and GeneRuler 3000 bp Plus DNA ladder (Thermo Scientific USA).

Table 4 Primers used in PCR.

Virus	Primers			Product size	Annealing temperature
HetPV2 (Vainio et al. 2011a)	7RPmidF1 & 7RPendR1	Forward primer Reverse primer	TAT GCC CAC GGA ACA ATA CA CAT ACC GTT GAG GTT GGT GT	544 bp	57 °C
HetPV4 (Vainio et al. 2011b)	RTendF & RTedR	Forward primer Reverse primer	TGA TCT CGA CCC AAA AGT CC CGC AAG GAG GAT GAG AAA AG	441 bp	57 °C
HetRV6 (Vainio et al. 2012)	CurFiAus & CurRe2	Forward primer Reverse primer	TTG AAT CAC CTG GAC CGT TT CAT CAA CCC ATT ATC CAG GT	714 bp	55 °C
HetPV7 (Vainio et al. 2015a)	7R242SpecF & NPRev431	Forward primer Reverse primer	CAA TCC TCG CTA GAG CTT CA TCG ACG GGT GTA ACT TCT TG	469 bp	57 °C
HetPV9 (Vainio et al. 2015b)	7R242CCFor & 7R422CCRev	Forward primer Reverse primer	AGC GTC GTA GAA GTC GGA AA AAG CTT TCT CGC TGC TCT TG	536 bp	57 °C
HetPV13 (Kashif et al. 2015)	HV3ConF1 & HV3ConRe1	Forward primer Reverse primer	ATG TTY TTC TGG CCY TTN TTC GCG ANG TGR TCG AAG TAG TA	608 bp	55 °C
HetPV16 (Hyder et al. 2018)	RHFor57 & RHRev596	Forward primer Reverse primer	TCC CAG CAC CTA CCC TGA CTT GTG CGC TTC AAC GAA	540 bp	53 °C

3.2.7 Somatic incompatibility test and genotype analysis

To make sure the culture in 3.2.2 and the following cells collected in 3.2.3 were not mixed with donor hyphae, two analysis methods were conducted, somatic incompatibility test and genotype analysis.

Somatic incompatibility test was done by carrying out pairing test on malt plate in two ways. One way is placing the original virus free fungal recipient on one side of the petri dish and the virus infected recipient on the other side of the petri dish, as is shown in Figure 4. There should be no demarcation line because the strains should be compatible. If the demarcation line shows, it means the virus infected recipient collected before was mixed with donor cells. The other way is placing the virus infected recipient on one side of the petri dish and the original fungal donor on the other side of the petri dish (Figure 5). There should be a demarcation line showing in the middle of the plate because the two isolates are not compatible, as is shown in Figure 5, indicated with an orange stripe. If no demarcation line shows, it means that the recipient collected was mixed with donor cells or the cells were only donor cells. Both ways were done in this thesis. Note that somatic incompatibility test is not accurate because sometimes demarcation line is not obvious or sometimes strange forms of mycelium appearance can be mistaken as demarcation line. Rather: it is not capable of revealing all incompatible strains if they are very closely related.

Genotype analysis was done by comparing the DNA fingerprints of all the recipient side's isolates after transmission experiment and the DNA fingerprints of the original donor. Only fungus strains that were successfully infected by virus were checked in genotype experiments. Recipient cells were collected on MOS (+) plates from 3.2.3. The original donor and the virus free recipient were inoculated onto MOS (+) plates too for collecting hyphae cells. Total fungal DNA was extracted from cultivated fungal mycelia as the protocol described by Vainio et al. (1998). If the DNA fingerprint of the recipient side's isolates are different from the donor and identical with the original recipient, the pure culture inoculated in 3.2.2 and cells collected in 3.2.3 are proven to be true recipient and therefore the transmission result is taken into account, otherwise the transmission result is not taken account.

Primers for genotype analysis is M13 minisatellite primer for DNA fingerprinting (Stenlid et al. 1994, Vainio et al. 2001). Its sequence is [GAG GGT GGC GGT TCT], and the annealing temperature is 48 °C for 1 min.

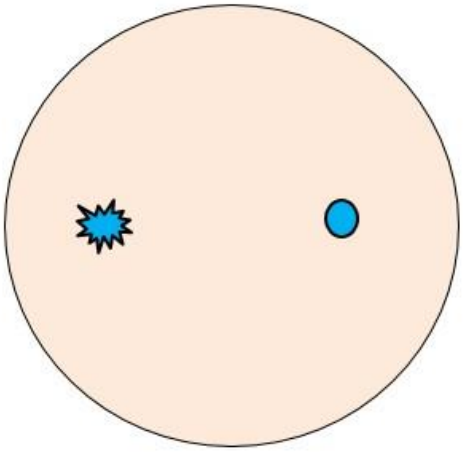

Figure 4 Somatic incompatibility test between virus infected recipient and virus free recipient. Blue irregular dot represents recipient fungus agar after virus transmission experiment that possibly contains viruses. Blue smooth dot represents the same recipient before transmission experiment and without viruses.

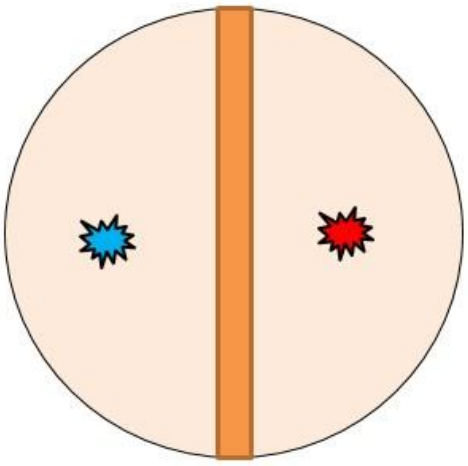

Figure 5 Somatic incompatibility test between virus infected recipient and donor. Blue irregular dot represents recipient agar after virus transmission experiment that possibly contains viruses. Red irregular dot represents donor agar containing five viruses. The orange stripe represents the demarcation line that is possible to show.

3.2.8 Growth rate experiments

To test how partitiviruses co-infection affect their new fungus hosts, a growth rate experiment was conducted. Since this study focuses on multiple virus transmission, only fungal recipients successfully infected by at least three viruses were chosen to be tested in the growth rate experiment. Virus free recipient fungal strains were tested as control group. Some fungal strains were also successfully transmitted by one or two viruses; however, they were not included in the growth rate experiments. All of the inoculations for the growth rate experiments were done on the same day.

Virus-containing recipient fungal strains and virus free recipient fungal strains were first pre-cultured at 20 °C for a week in order to obtain fresh mycelia for the growth rate experiments and to avoid temperature shock. Then a small piece of agar of multi-virus fungal recipient was plugged out using a pasteur pipette and inoculated onto the center of fresh 2% malt agar plate and incubated at 20 °C. Every 24 hours, a planimeter (Planix 10S Tamaya) was used to measure the growth area (cm^2) of the fungus until the growth area covered the whole plate. Each measurement was marked by drawing the circle of growth area, as in Figure 6. Five parallel replicates of each fungal strain were prepared in the growth rate experiment.

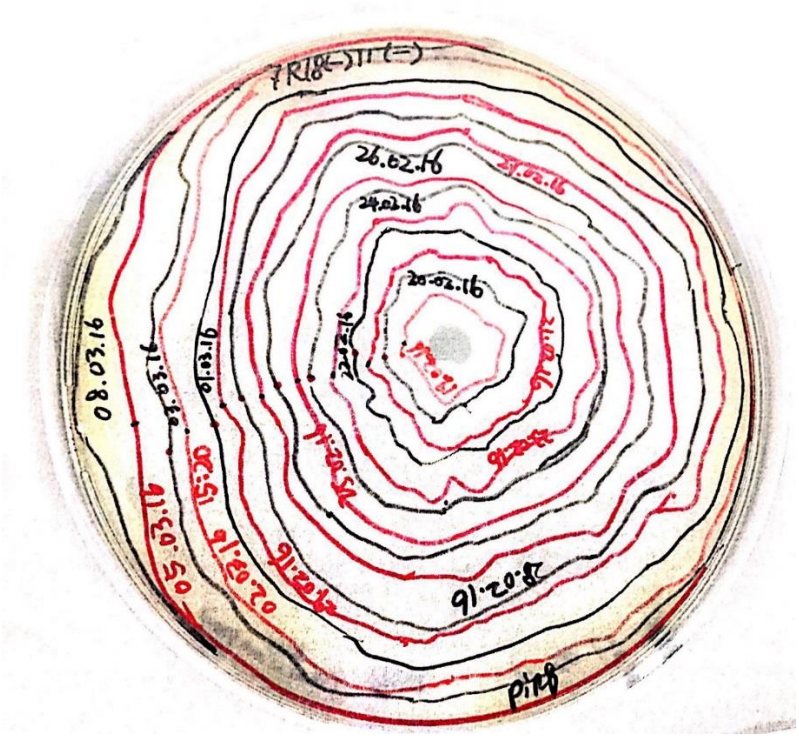

Figure 6 An example of growth rate experiment of fungal strain: virus free 7R18-11.

3.2.9 Data analysis

The original statistics of growth curve data are included in Appendix 5. The growth curves of infected fungal isolates are presented in the form of scatter plot chart. These figures were made by using Excel 2016. The average growth area values of five parallel plates were used to draw the scatter chart and the smooth lines, the standard deviation (stdev) values were used to add the error bars in the chart.

Growth curves were used to calculate the growth rate. The information of how many days it took the fungus to grow certain area on the growth curve can be exploited to calculate the growth rate. When fungus strains were inoculated onto new plates, they were facing a new environment, so the first stage of their growth was rather slow because they needed time to adapt to new environment. And when they were about to grow to the edge of the plate, their growth rate was also affected due to lack of nutrients and space. In order to make the growth rate results more accurate, here we only analyze

the logarithmic growth rate, so only growth area from 10 cm^2 to 40 cm^2, or 10 cm^2 to 50 cm^2, are included into the calculation, 10-50 cm^2 data were included to make a comparison with 10-40cm^2 data. The growth rate was calculated by formula: 1) growth rate between 10 cm^2 to 40 cm^2= (40-10)/ (number of days to grow to 40 cm^2- number of days to grow to 10 cm^2) cm^2/day. 2) growth rate between 10 cm^2 to 50 cm^2= (50-10)/ (number of days to grow to 50 cm^2- number of days to grow to 10 cm^2) cm^2/day. The original statistics of growth rate data are included in Appendix 6.

Another way to calculate the growth rate would have been to consider the growth rate from certain day for example day 3 to another day for example day 9, but fungus strains grow at different pace and it might take 20 days for one isolate to grow to the edge or only 8 days for another isolate to fully cover the plate, so it is hard to decide a standard starting date and an ending date. Eventually, to define growth rate the first method was used.

The average logarithmic growth rate is presented by bar plot made from Excel 2016. As there are five parallel plates of each fungus in the growth rate experiment, there are five growth curves too, so five growth rate values were calculated. The average growth rate value of the five was used as the final average logarithmic growth rate of each fungus in the growth rate bar plot, and the standard deviation value was added as error bar.

To determine whether there is a significant difference between the growth rate of virus free fungus and multi-virus infected fungus, a data analysis method in Microsoft 2016 Excel-T test was used. The way to run a t test is to use excel-data analysis-t test- “Paired Two Sample for Means”. The growth rate of virus free fungus was always used as variable 1 and the growth rate of multiple viruses infected fungus was used as variable 2. All comparisons are between the viruses infected growth rate and the virus free growth rate. P value (two-tail) of 0.05 is used for statistical significance. In figures, $0.01<p\text{-value}<0.05$ is represented by an asterisk *, $0.001<p\text{-value}<0.01$ is represented by two asterisks **, p-value less than 0.001 is represented by three asterisks ***. The original statistics of p values are included in Appendix 7.

4 Results

4.1 Virus transmission results

This is the basic virus transmission results which are based on RT-PCR and gel electrophoresis before the somatic incompatibility test and genotype analysis.

In the first part of the transmission result, both donors are *H. parviporum*, and all four recipients are *H. parviporum*. As shown in Table 5 many viruses were transmitted successfully. Only one virus, HetPV7 was not transmitted at all. Virus HetPV2 and HetPV13 were transmitted most often, followed closely by HetPV16 and HetRV6. Next ones were HetPV4 and HetPV9, each transmitted twice out of eight transmission experiments. There are double virus transmissions and many multiple virus transmissions which is what this thesis focuses on. As is shown in Table 5, the blue columns indicate invalid results as the fungus was checked by PCR and gel electrophoresis twice but with different results. The results of the blue ones are considered to be inconsistent and therefore are not included among the final successful transmissions in 4.4.

In the second part of the transmission result, both donors are *H. parviporum*, all six recipients are *H. annosum*. Very different from the first part of the transmission result, as indicated in Table 6, in most cases the viruses were not successfully transmitted. Only two viruses, HetPV13 and HetPV16 were transmitted to new hosts. No multiple virus transmission occurred, only one double virus transmission took place and all the other transmissions were single virus transmission. In Table 6, blue column was proved to be invalid after somatic incompatibility result and genotype result because both tests showed that the agar plugged out and inoculated in method 3.2.2 was not from recipient but from donors, so it was excluded from the result in 4.4.

Figure 7 is an example gel picture showing the virus positive recipient and virus negative recipient. Another discover from the transmission experiment is that, one recipient fungus, 7R18-11, showed so little growth on the transmission plate, as is shown in Figure 8. All the other recipients occupied about half of the plate after

transmission experiment, 7R18-11 was the only recipient that grew only a small brown circle. In all the four plates which have 7R18-11, it showed the same situation.

Table 5 Transmission result 1, *H. parviporum* to *H. parviporum*. "(1)" and "(2)" represent replicate one and replicate two respectively in the transmission experiment. "+" means the virus transmission is successful and "-" means virus transmission was not successfully. Blue column is invalid result.

H. parviporum recipient fungal strains	Viruses from Donor 1					Viruses from Donor 2				
	HetP V2	HetP V4	HetR V6	HetP V13	HetP V16	HetP V2	HetR V6	HetP V7	HetP V9	HetP V13
RK5A (1)	+	-	+	+	-	+	-	-	-	+
RK5A (2)	+	-	+	+	-	-	-	-	-	-
7R18-11 (1)	+	+	-	+	+	-	-	-	+	+
7R18-11 (2)	+	+	+	+	+	+	+	-	+	+
SB10.16 (1)	-	-	-	-	-	+	-	-	-	+
SB10.16 (2)	+	-	+	+	+	+	+	-	-	+
EV0789 (1)	+	-	-	+	+	+	-	-	-	+
EV0789 (2)	+	-	+	+	+	+	+	-	-	+

Table 6 Transmission result 2, *H. parviporum* to *H. annosum*. "(1)" and "(2)" represent replicate one and replicate two respectively in the transmission experiment. "+" means the virus transmission is successful and "-" means virus transmission was not successfully. Blue column is invalid result.

H. annosum recipient fungal strains	Viruses from Donor 1					Viruses from Donor 2				
	HetP V2	HetP V4	HetR V6	HetP V13	HetP V16	HetP V2	HetR V6	HetP V7	HetP V9	HetP V13
S49-5 (1)	-	-	-	-	-	-	+	-	-	-
S49-5 (2)	-	-	-	+	+	-	-	-	-	+
03021 (1)	-	-	-	-	-	-	-	-	-	-
03021 (2)	-	-	-	-	-	-	-	-	-	+
TPHA5·31 (1)	-	-	-	-	-	-	-	-	-	-
TPHA5·31 (2)	-	-	-	-	-	-	-	-	-	+
T60-9 (1)	-	-	-	-	-	-	-	-	-	-
T60-9 (2)	-	-	-	-	-	-	-	-	-	-
93173 (1)	-	-	-	-	+	-	-	-	-	-
93173 (2)	-	-	-	-	-	-	-	-	-	+
RKON1.60 (1)	-	-	-	-	-	-	-	-	-	-
RKON1.60 (2)	-	-	-	-	-	-	-	-	-	-

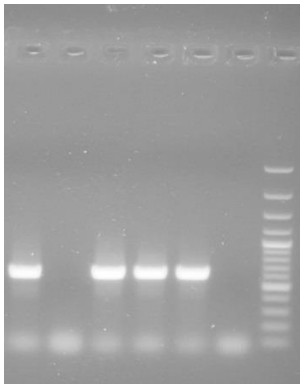

Figure 7 An example gel picture of virus positive and virus negative recipient.

Figure 8 Transmission experiment between donor MV2 and recipient 7R18-11. The brown circle is the area of 7R18-11 mycelium area after three months' growth.

4.2 Somatic incompatibility test

The result of somatic incompatibility test shows that majority of the recipients' side isolates are true recipients' cells (Table 7). It was expected that on the pairing test plates, between the recipient-side-isolate and the control virus free recipient, mycelium from both sides grow into each other without a demarcation line. In contrast, on the pairing test plates between the recipient-side isolate and the donor, mycelium from both sides should form a demarcation line in the middle.

Only two recipients' side isolates are not from recipients, these results are emphasized by brown background in Table 7. They are either mixed cells with both donor and recipients' cells, or only donor's cells. In Replicate 1, only one paring: S49-5 infected by MV2 suggests that the donor but not the recipient mycelium was isolated from the recipient side. However, genotype analysis is still necessary to prove whether this is a true fault. In Replicate 2, one recipient EV0789 infected by MV1 suggests that the donor but not the recipient mycelium was isolated from the recipient side. With two recipients, it was hard to interpret whether there is a demarcation line or not. They are emphasized by green background color in Table 7. Still, genotype analysis was further conducted to confirm whether these are true faults.

Somatic incompatibility test results were checked by genotype test later. Genotype analysis is more accurate compared to somatic incompatibility test. Whether a recipient side isolate is truly from the recipient, is based on genotype analysis result.

Table 7 Somatic incompatibility test results. "－" means demarcation lines was not seen on the plate, "＋" means demarcation lines was seen on the plate. Brown shading is used to emphasize that the recipient-side-isolate is not true recipient. When it is hard to tell whether there is a demarcation line, it is indicated by the green background in the table.

Donor in 3.2.1	Recipient-side isolate (after transmission experiment) vs. Recipient (virus free)	Demarcation line		Recipient-side isolate (after transmission experiment) vs. Donor	Demarcation line	
		Replicate 1	Replicate 2		Replicate 1	Replicate 2
MV1	S49-5 vs. S49-5	－	－	S49-5 vs. MV1	＋	＋
	03021 vs. 03021	－	－	03021 vs. MV1	＋	＋
	HA5.31 vs. HA5.31	－	－	HA5.31vs. MV1	＋	＋
	T60-9 vs. T60-9	－	－	T60-9 vs. MV1	＋	＋
	93173 vs. 93173	－	－	93173 vs. MV1	＋	＋
	RK5A vs. RK5A	－	－	RK5A vs. MV1	＋	＋
	RKON1.60 vs. RKON1.60	－	－	RKON1.60 vs.MV1	＋	＋
	7R18(-)11 vs. 7R18(-)11	－	－	7R18(-)11 vs. MV1	＋	＋
	SB10.16 vs. SB10.16	－	－	SB10.16 vs. MV1	＋	＋
	EV0789 vs. EV0789	－	＋	EV0789 vs. MV1	＋	－
MV2	S49-5 vs. S49-5	＋	－	S49-5 vs. MV2	－	＋
	03021 vs. 03021	－	－	03021 vs. MV2	＋	＋
	HA5.31 vs. HA5.31	－	－	HA5.31vs. MV2	＋	＋
	T60-9 vs. T60-9	－	－	T60-9 vs. MV2	＋	＋
	93173 vs. 93173	－	－	93173 vs. MV2	＋	＋
	RK5A vs. RK5A	－	Hard to tell	RK5A vs. MV2	＋	Hard to tell
	RKON1.60 vs. RKON1.60	－	－	RKON1.60 vs.MV2	＋	＋
	7R18(-)11 vs. 7R18(-)11	－	－	7R18(-)11 vs. MV2	＋	＋
	SB10.16 vs. SB10.16	－	－	SB10.16vs. MV2	＋	＋
	EV0789 vs. EV0789	－	Hard to tell	EV0789vs. MV2	＋	Hard to tell

4.3 Genotype analysis

Genotype analysis proved that almost all recipients' side isolates are real recipients' cells. The only exception is the isolate from recipient S49-5's side in replicate one transmission experiment, where it was infected by MV2. It is actually the same with donor fungus strain, as is emphasized by yellow background in Table 8. So, the transmission result of this pairing: S49-5 infected by MV2 in replicate one is not a true result. It was removed from the final transmission results in 4.4.

The phenotype result agrees with most of the genotype result, including on fungus S49-5 infected by donor MV2 in replicate one (see Table 7 and Table 8). However, on fungus EV0789 infected by donor MV1 in replicate two, the genotype results show the recipient fungus EV0789 was not contaminated by donor and the transmission did happen while in phenotype results the donor and recipient could not be distinguished. The final result of EV0789 is based on genotype result because it is more accurate. The results in Table 7 that are hard to tell were tested by genotype results to be true recipients (Table 8). Figure 9 is an example synergel picture of donor and recipient fingerprinting different from each other.

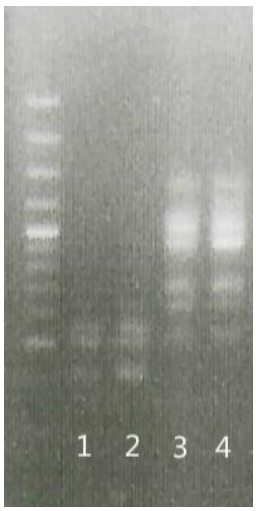

Figure 9 An exemplar gel picture of donor and recipient differed from each other. 1 and 2 are donor MV1, 1 is original DNA of MV1, 2 is 1:10 diluted DNA of MV1. 3 and 4 are recipient 93173 DNA, 3 is original DNA of 93713, 4 is 1:10 diluted 93173 DNA.

Table 8 Genotype results of the infected recipient fungus. “+” means that the fungal agar inoculated in 3.2.2 and cells collected in 3.2.3 were actually the same as the recipient, and “–” means opposite and is emphasized by yellow shading.

Donor	Recipient (infected and not infected)	Replicate 1 genotype	Replicate 2 genotype
MV1	S49-5	+	+
	03021		
	HA5.31		
	T60-9		
	93173	+	
	RK5A	+	+
	RKON1.60		
	7R18(-)11	+	+
	SB10.16		+
	EV0789	+	+
MV2	S49-5	–	+
	03021		+
	HA5.31		+
	T60-9		
	93173		
	RK5A	+	
	RKON1.60		
	7R18(-)11	+	+
	SB10.16	+	+
	EV0789	+	+

4.4 Final combined results of transmission rate

The final transmission results were first testified by PCR using specific virus primers and following gel electrophoresis, and then corrected by results of the somatic incompatibility test and genotype analysis. The inconsistent PCR results are removed, and the invalid results of somatic incompatibility test and genotype test were removed too.

In many cases, multiple virus transmission was verified which demonstrates multiple mycovirus transmission is possible between *Heterobasidion* fungal strains, but it only happened from *H. parviporum* to *H. parviporum* (Table 9 and Table 10). The transmission success rate from *H. parviporum* to *H. parviporum* of all viruses are obviously higher than that from *H. parviporum* to *H. annosum*. In fact, virus transmission is very rare from *H. parviporum* to *H. annosum* (Table 10). Apart from that, the transmission rate is different for each virus, the gap between the highest rate and the lowest rate is big (Table 9 and Table 10). HetPV2 and HetPV13 showed the highest transmission rate from *H. parviporum* to *H. parviporum*: 87.5% of HetPV2 from Donor 1 to *H. parviporum* recipients, 75% of HetPV13 from Donor 1 to *H. parviporum* recipients; 75% of HetPV2 from Donor 2 to *H. parviporum* recipients and 87.5% of HetPV13 from Donor 2 to *H. parviporum* recipients. HetPV16 also showed relatively high transmission rate from Donor 1 to *H. parviporum* recipients, which is 62.5%. Following HetPV16 are HetRV6, HetPV4 and HetPV9. HetPV7 was the only virus not transmitted at all, no matter what species the recipients are. HetPV13 and HetPV16 showed highest transmission rate (Table 10) from *H. parviporum* to *H. annosum*, although much lower compared to their transmission rate from *H. parviporum* to *H. parviporum* (Table 9). They are also the only two viruses that were transmitted from *H. parviporum* to *H. annosum*.

Table 9 Final transmission result 1, *H. parviporum* to *H. parviporum*. "(1)" and "(2)" represent replicate one and replicate two respectively in the transmission experiment. "+" means the virus transmission is successful and is emphasized with yellow shading. "-" means the virus transmission was not successful.

H. parviporum recipient fungal strains	Viruses from Donor 1					Viruses from Donor 2				
	HetP V2	HetP V4	HetR V6	HetP V13	HetP V16	HetP V2	HetR V6	HetP V7	HetP V9	HetP V13
RK5A (1)	+	-	+	+	-	+	-	-	-	+
RK5A (2)	+	-	-	-	-	-	-	-	-	-
7R18-11 (1)	+	+	-	+	+	-	-	-	+	+
7R18-11 (2)	+	+	+	+	+	+	+	-	+	+
SB10.16 (1)	-	-	-	-	-	+	-	-	-	+
SB10.16 (2)	+	-	+	+	+	+	+	-	-	+
EV0789 (1)	+	-	-	+	+	+	-	-	-	+
EV0789 (2)	+	-	+	+	+	+	+	-	-	+
Transmission rate (%)	87.5	25	50	75	62.5	75	37.5	0	25	87.5

Table 10 Final transmission result 2, *H. parviporum* to *H. annosum*. "(1)" and "(2)" represent replicate one and replicate two respectively in the transmission experiment. "+" means the virus transmission is successful and is emphasized with yellow shading. "-" means the virus transmission was not successful.

H. annosum recipient fungal strains	Viruses from Donor 1					Viruses from Donor 2				
	HetP V2	HetP V4	HetR V6	HetP V13	HetP V16	HetP V2	HetR V6	HetP V7	HetP V9	HetP V13
S49-5 (1)	-	-	-	-	-	-	-	-	-	-
S49-5 (2)	-	-	-	+	+	-	-	-	-	+
03021 (1)	-	-	-	-	-	-	-	-	-	-
03021 (2)	-	-	-	-	-	-	-	-	-	+
TPHA5·31 (1)	-	-	-	-	-	-	-	-	-	-
TPHA5·31 (2)	-	-	-	-	-	-	-	-	-	+
T60-9 (1)	-	-	-	-	-	-	-	-	-	-
T60-9 (2)	-	-	-	-	-	-	-	-	-	-
93173 (1)	-	-	-	-	+	-	-	-	-	-
93173 (2)	-	-	-	-	-	-	-	-	-	+
RKON1.60 (1)	-	-	-	-	-	-	-	-	-	-
RKON1.60 (2)	-	-	-	-	-	-	-	-	-	-
Transmission rate (%)	0	0	0	8.33	16.7	0	0	0	0	33.3

4.5 Growth curves

Based on final transmission result Table 9 and Table 10, the recipient *Heterobasidion* fungus infected by at least 3 viruses are as follows, all together nine combinations of fungus and viruses, as is shown in Table 11. As there were 5 parallel growth rate experiments of each combination, the average numbers of the five parallel growth area were used in the growth data curves. Blue curve is always used to represent the virus free isolate.

Table 11 recipient and multiple virus combination

Recipient infected by more than three viruses	The viruses infected the fungus
RK5A	HetPV2, HetRV6 and HetPV13
7R18-11	HetPV2, HetRV6, HetPV9 and HetPV13
7R18-11	HetPV2, HetPV4, HetPV13 and HetPV16
7R18-11	HetPV2, HetPV4, HetRV6, HetPV13 and HetPV16
SB10.16	HetPV2, HetRV6 and HetPV13
SB10.16	HetPV2, HetRV6, HetPV13 and HetPV16
EV0789	HetPV2,HetPV13 and HetPV16
EV0789	HetPV2,HetRV6 and HetPV13
EV0789	HetPV2,HetRV6,HetPV13 and HetPV16

4.5.1 Growth curves of RK5A

As is shown in Figure 10, there are two growth curves representing the virus free RK5A using blue curve and viruses infected RK5A using red curve separately. Day 0 is the day the two isolates were inoculated. It took the virus free RK5A 10 days to reach maximal area. It took the virus-infected RK5A 11 to 12 days to reach the maximal area (Figure 10). The growth curve of viruses infected RK5A is below the virus free one all the time, which means the viruses infected RK5A grew slower than the virus free one. Error bars represent the standard deviation values of five parallel growth rate experiments.

In the first four days, the two curves are very close, which means the two isolates show similar ability to adapt to new environment.

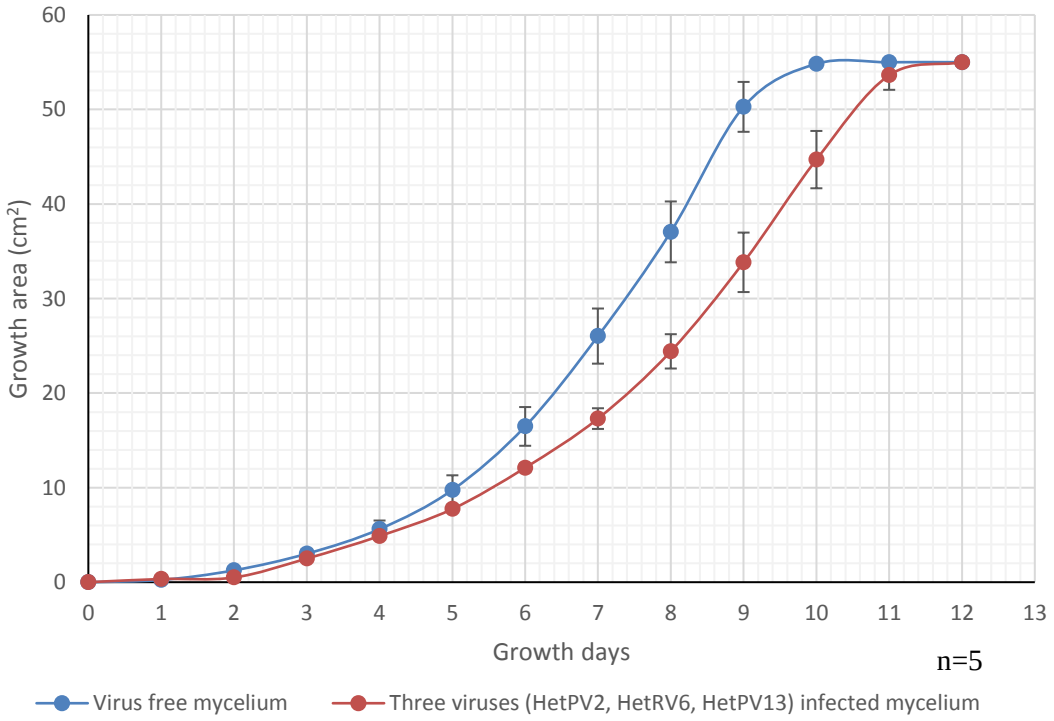

Figure 10 Growth curves of RK5A with and without multiple viruses.

4.5.2 Growth curves of 7R18-11

Based on Figure 11, the growth curve of virus free 7R18-11 in blue is quite different from the three other growth curves. It only took the virus-infected ones 10 to 12 days to reach maximal area, but 20 days for the virus free 7R18-11 to reach there. It can be concluded that the virus free 7R18-11 grew much slower than viruses infected 7R18-11. As to other growth curves, although their starting phase took different time, their growth trend is quite similar, looked quite parallel in Figure 11, which means their growth rate might be quite similar. The red curve showed that isolate 7R18-11 infected by 4 viruses (HetPV2, HetRV6, HetPV9 and HetPV13) used least time to adapt to new environment. Among all the other fungal strains in growth rate experiment, no matter virus free or viruses infected isolates, it only took them around 10 days to reach maximal area. Combined with the fact that 7R18-11 showed so little growth in the transmission experiment, it is possible that it degenerated somehow.

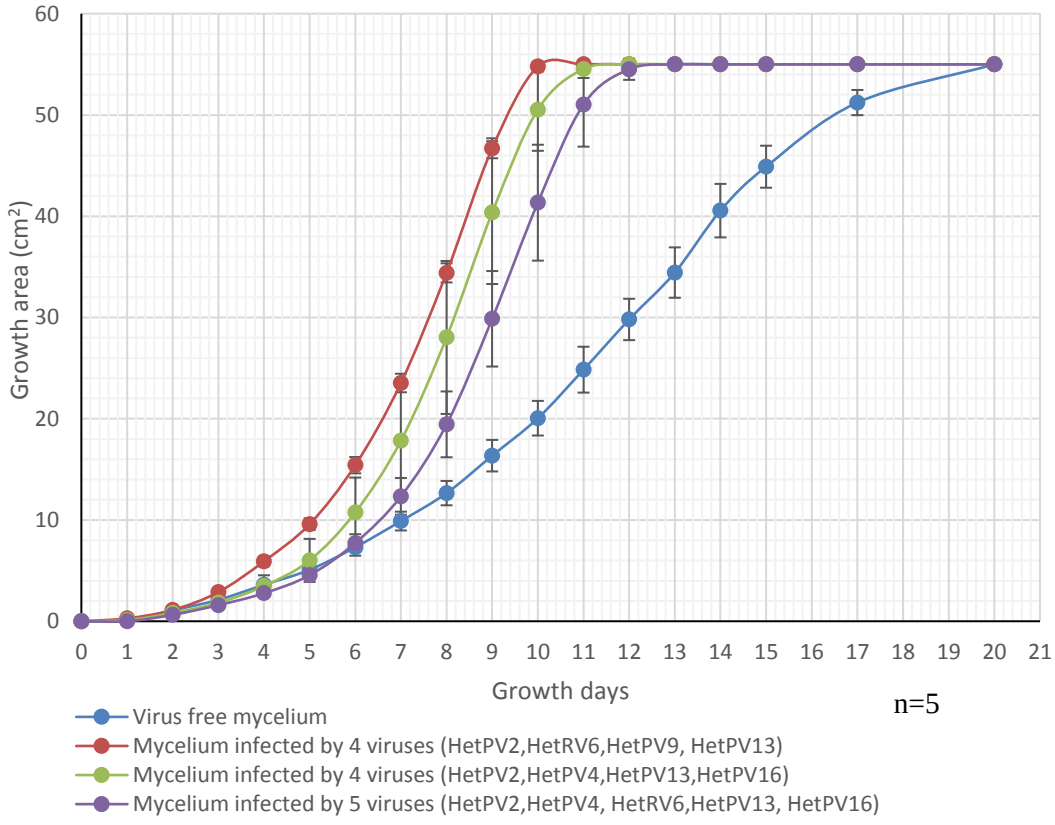

Figure 11 Growth curves of 7R18-11.

4.5.3 Growth curves of SB10.16

As is shown in Figure 12, all three growth curves of SB10.16 are very similar. It took all of them about the same days (around 8 to 9 days) to reach maximal area (Figure 12). Their growth trend is also similar. It is hard to tell which one is the fastest only by the curves. Their ability to adapt to new condition is also very close. Day 0 is the day all isolates were inoculated. Error bars represent the stdev values of five parallel growth rate experiments.

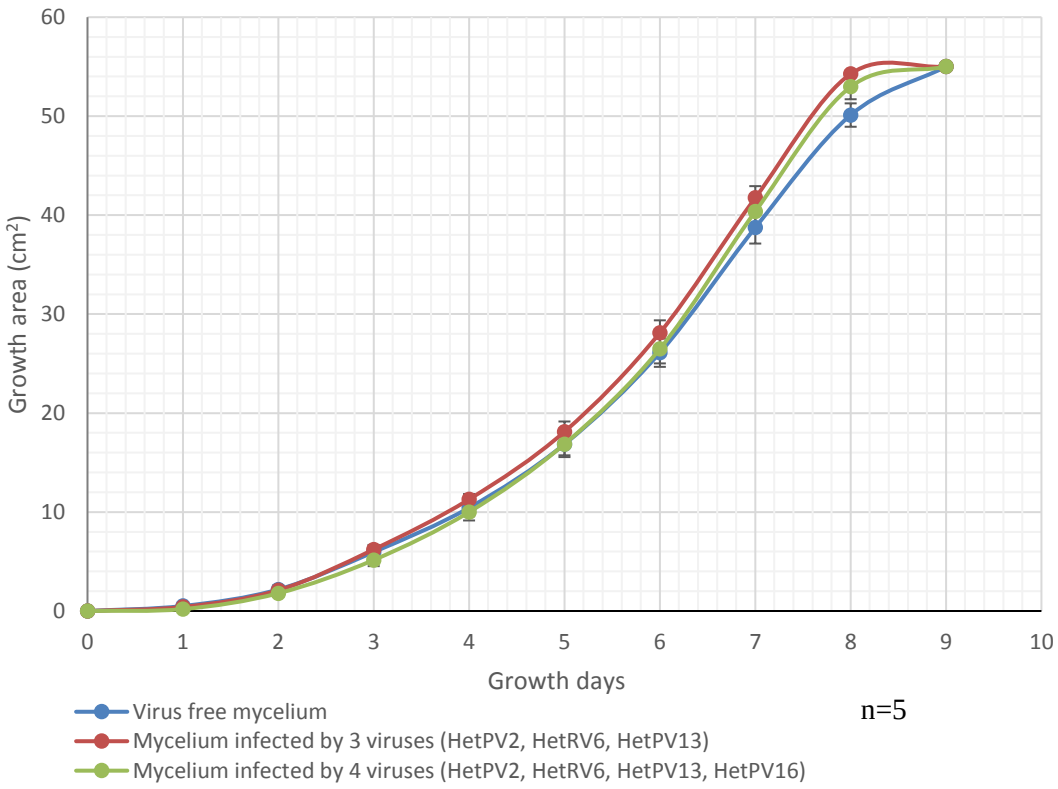

Figure 12 Growth curves of SB10.16.

4.5.4 Growth curves of EV0789

For all four isolates of EV0789 it took 9 days to reach their maximal growth area (Figure 13). Their growth rates are similar, and it cannot be read through the growth curves which one is the fastest. Red curve representing EV0789 infected by HetPV2, HetPV13 and HetPV16 used less time to adapt to new environment. It is also always vertically always higher than the other curves. However, the growth rate of red curve is not necessarily higher than those of other curves.

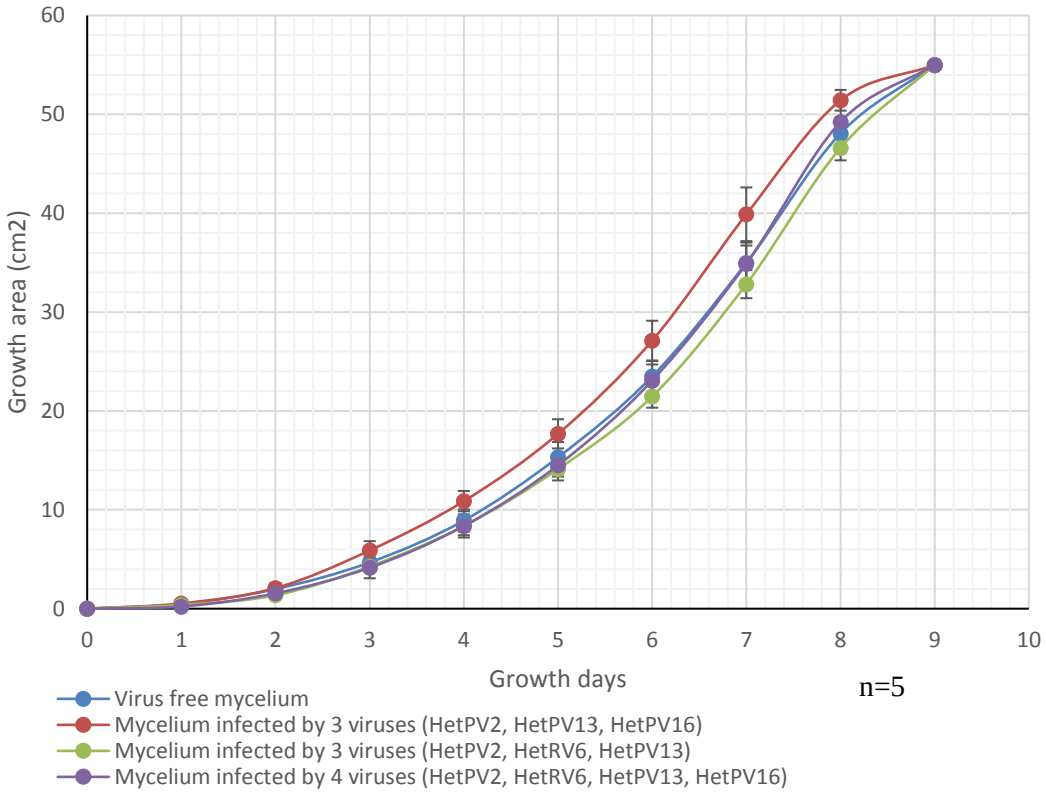

Figure 13 Growth curves of EV0789.

4.6 Growth rate of recipient *Heterobasidion* fungus

The growth rate of each fungal strain is presented in bar plot. Growth rate shown in the bar plots is the average growth rate of five parallels, stdev values are used as error bars. The growth rates of each isolate from 10 cm^2 to 40 cm^2 and from 10 cm^2 to 50 cm^2 are presented separately in one bar plot. Blue bar is always the growth rate of virus free isolate.

4.6.1 Growth rate of RK5A

The growth rate of virus free RK5A and 3 viruses (HetPV2, HetRV6 and HetPV13) infected RK5A is shown in Figure 14. Here we compare the growth rate of virus free RK5A and of RK5A infected by 3 viruses from 10 cm^2 to 40 cm^2, also the growth rate of virus free RK5A and of 3 viruses infected RK5A from 10 cm^2 to 50 cm^2.

Blue column represents the average logarithmic growth rate of virus free RK5A. Red column stands for the growth rate of viruses infected RK5A. Based on Figure 14, the growth rate of RK5A decreased a lot when it was infected by multiple viruses (HetPV2, HetRV6 and HetPV13), in the interval of both 10 to 40 cm^2 and 10 to 50 cm^2.

When the average logarithmic growth rate is calculated in the interval of 10-40 cm^2, the growth rate of viruses infected RK5A decreased around 2 cm^2 per day, the difference between the growth rate of viruses infected RK5A and the growth rate of virus free RK5A is statistically significant ($0.001<p<0.01$).

When the average logarithmic growth rate is calculated in the interval of 10-50 cm^2, the growth rate of viruses infected RK5A decreased around 2 cm^2 per day, the difference between the growth rate of viruses infected RK5A and the growth rate of virus free RK5A is also statistically significant ($p<0.001$).

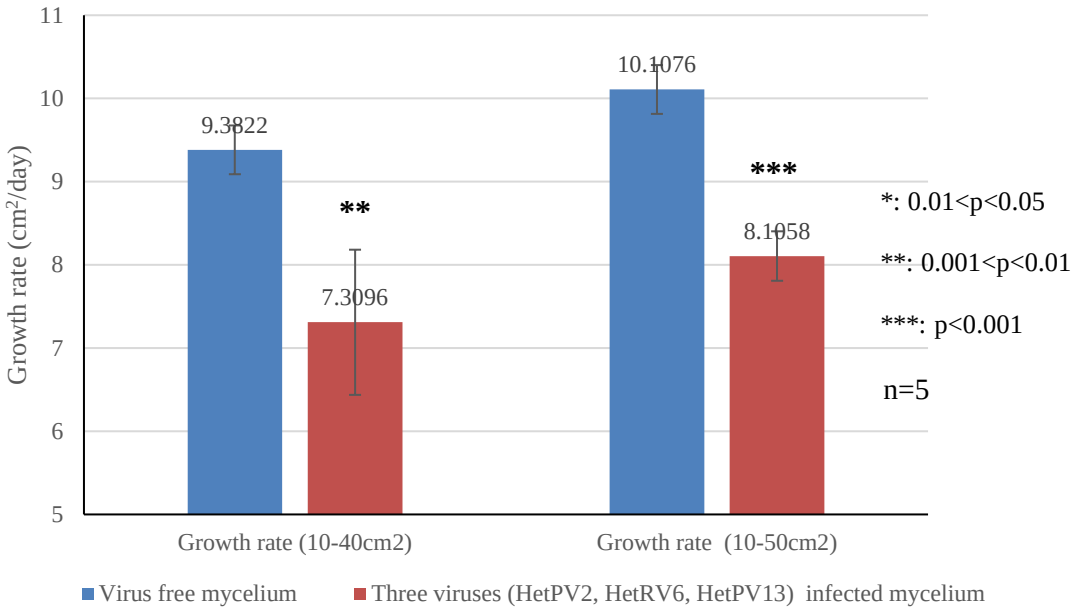

Figure 14 Average logarithmic growth rate of RK5A from 10-40 cm^2 and from 10-50 cm^2.

4.6.2 Growth rate of 7R18-11

As there are three isolates of 7R18-11 infected by multiple viruses, there are three individual comparisons, all against the virus free 7R18-11: 1) the growth rate of 7R18-11 infected by 4 viruses (HetPV2, HetRV6, HetPV9, HetPV13) vs the growth rate of virus free 7R18-11; 2) the growth rate of 7R18-11 infected by 4 viruses (HetPV2, HetPV4, HetPV13, HetPV16) vs the growth rate of virus free 7R18-11; 3) the growth rate of 7R18-11 infected by 5 viruses (HetPV2, HetPV4, HetRV6, HetPV13, HetPV16) vs the growth rate of virus free 7R18-11.

Based on Figure 15, the growth rate of viruses infected 7R18-11 increased a lot compared to virus free 7R18-11. The growth rate of all three isolates infected by multiple viruses is a little more than twice the growth rate of virus free 7R18-11. All three isolates of 7R18-11 infected by multiple viruses show obvious growth rate increase in both calculated zone (10-40 cm^2 and 10-50 cm^2). As p values of all three isolates' growth rates are less than 0.001, the growth rate of the viruses infected three isolates is statistically significantly higher than the growth rate of virus free isolate.

The growth rate of 7R18-11 infected by HetPV2, HetPV4, HetPV13 and HetPV16 is the highest among all isolates. 7R18-11 infected by HetPV2, HetRV6, HetPV9 and HetPV 13 and 7R18-11 infected by HetPV2, HetPV4, HetRV6, HetPV13 and HetPV16 have close growth rates.

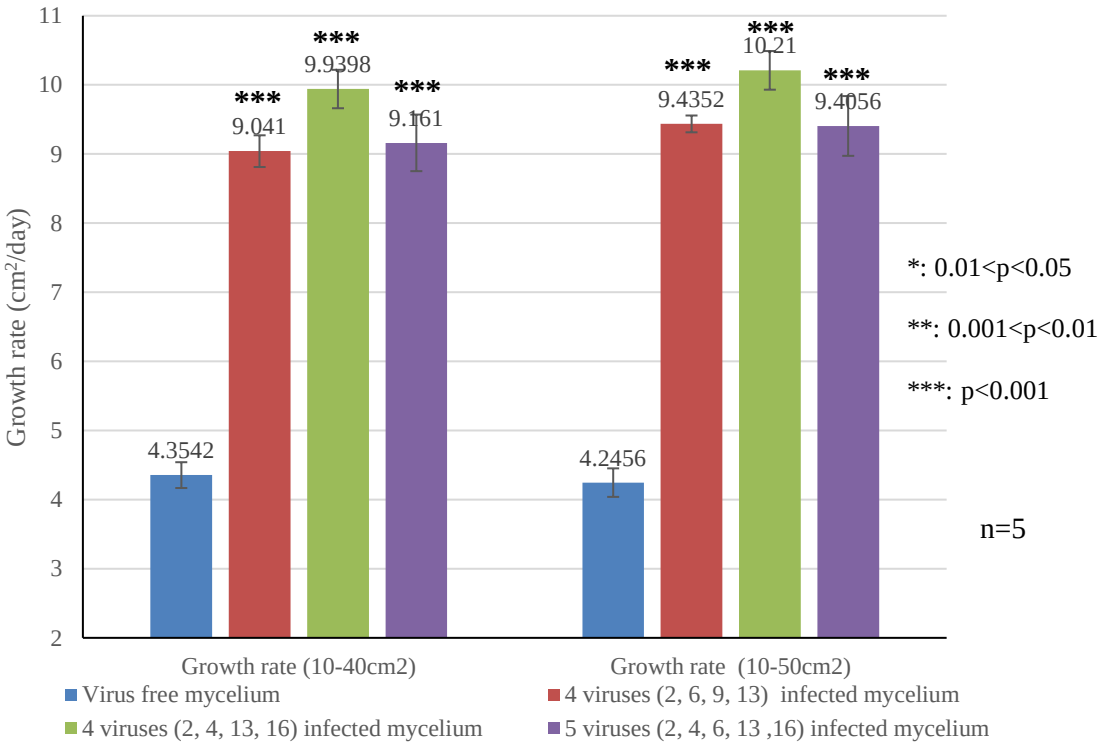

Figure 15 Average logarithmic growth rate of 7R18-11 from 10-40 cm^2 and from 10-50 cm^2. Numbers in brackets of the legends is short for the virus number. 2=HetPV2, 4=HetPV4, 6=HetRV6, 9=HetPV9, 13=HetPV13, 16=HetPV16.

4.6.3 Growth rate of SB10.16

As there are two isolates of SB10.16 infected by multiple viruses, there are two comparisons made: 1) the growth rate of 3 viruses (HetPV2, HetRV6, HetPV13) infected SB10.16 vs the growth rate of virus free SB10.16; 2) the growth rate of 4 viruses (HetPV2, HetRV6, HetPV13, HetPV16) infected SB10.16 vs the growth rate of virus free SB10.16.

In Figure 16, it is shown that multiple virus infection cause SB10.16 to grow faster than

virus free isolate.

When the average logarithmic growth rate is calculated in the interval of 10-40 cm^2, the growth rate of SB10.16 infected by HetPV2, HetRV6 and HetPV13 is only slightly higher than the growth rate of virus free SB10.16. There is no statistically significant difference between them (p value>0.05). The growth rate of SB10.16 infected by HetPV2, HetRV6, HetPV13 and HetPV16 is less than 1 cm^2/day higher than the growth rate of virus free SB10.16. The difference is statistically significant (0.01<p< 0.05).

When the average logarithmic growth rate is calculated in the interval of 10-50 cm^2, the growth rate of SB10.16 infected by HetPV2, HetRV6 and HetPV13 is only slightly higher than growth rate of virus free SB10.16. There is statistically significant difference between them (0.01<p value<0.05). The growth rate of SB10.16 infected by HetPV2, HetRV6, HetPV13 and HetPV16 is around 1 cm^2/day higher than the growth rate of virus free SB10.16. The difference is statistically significant (0.01<p< 0.05).

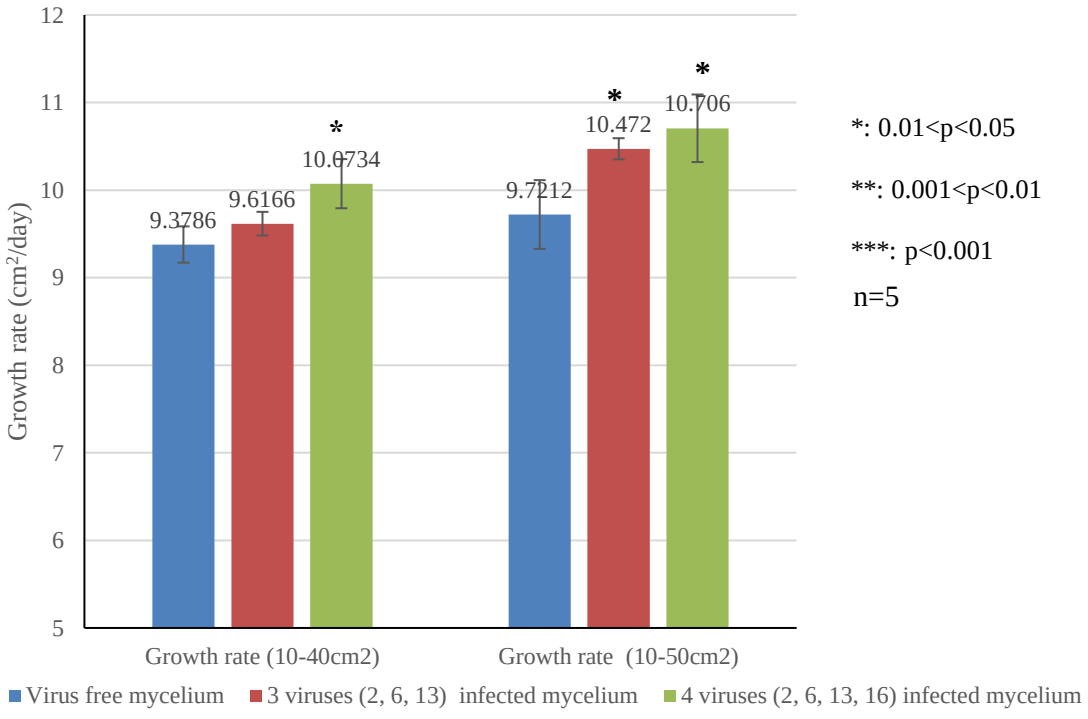

Figure 16 Average logarithmic growth rate of SB10.16 from 10-40 cm^2 and from 10-50 cm^2. Numbers in brackets of the legends is short for the virus number. 2=HetPV2, 6=HetRV6, 13=HetPV13, 16=HetPV16.

4.6.4 Growth rate of EV0789

As there are three isolates infected by multiple viruses, there are three comparisons made here: 1) the growth rate of EV0789 infected by 3 viruses (HetPV2, HetPV13 and HetPV16) vs the growth rate of virus-free EV0789; 2) the growth rate of EV0789 infected by 3 viruses (HetPV2, HetRV6 and HetPV13) vs the growth rate of virus-free EV0789; 3) the growth rate of EV0789 infected by 4 virus (HetPV2, HetRV6, HetPV13 and HetPV16) vs the growth rate of virus-free EV0789.

As is shown in Figure 17, when the average logarithmic growth rate is calculated in the interval of 10-40 cm^2, the growth rate of EV0789 infected by HetPV2, HetPV13 and HetPV16 is very close to the growth rate of virus free EV0789. There is no statistically significant difference between them (p value>0.05). The growth rate of EV0789 infected by HetPV2, HetRV6 and HetPV13 is close to the growth rate of virus free EV0789 too. The difference is not statistically significant ($p>0.05$). The growth rate of EV0789 infected by HetPV2, HetRV6, HetPV13 and HetPV16 is slightly higher than the growth rate of virus free EV0789, but the difference is not statistically significant different ($p>0.05$).

As is shown in Figure 17, when the average logarithmic growth rate is calculated in the interval of 10-50 cm^2, the growth rate of EV0789 infected by HetPV2, HetPV13 and HetPV16 is very close to the growth rate of virus free EV0789. There is no statistically significant difference between them (p value>0.05). The growth rate of EV0789 infected by HetPV2, HetRV6 and HetPV13 is close to the growth rate of virus free EV0789 too. The difference is not statistically significant ($p>0.05$). The growth rate of EV0789 infected by HetPV2, HetRV6, HetPV13 and HetPV16 is slightly higher than the growth rate of virus free EV0789, and the difference is statistically significant different ($p<0.001$).

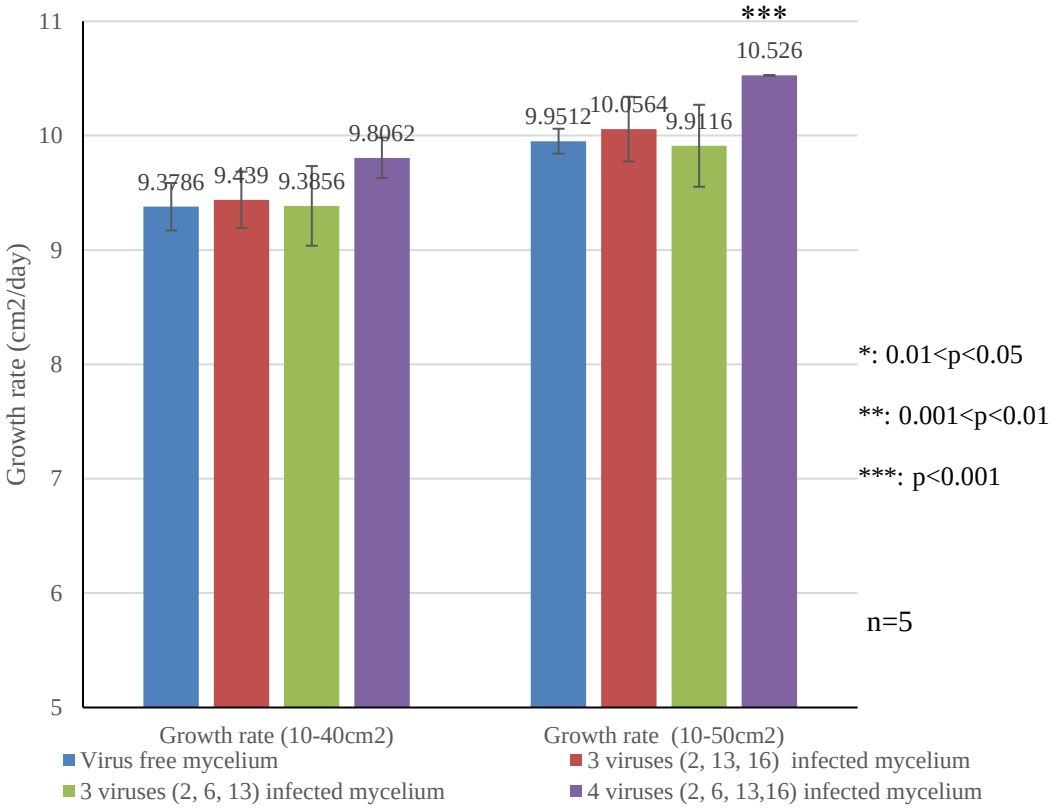

Figure 17 Average logarithmic growth rate of EV0789 from 10-40 cm^2 and from 10-50 cm^2. Numbers in brackets of the legends is short for the virus number. 2=HetPV2, 6=HetRV6, 13=HetPV13, 16=HetPV16.

4.6.5 Average growth rate difference in percentage

To make the difference of growth rates clearer, the differences were presented as percentage as is shown in Table 12 and Table 13. When growth rate is calculated in 10-40 cm^2 zone, multiple virus infection caused variable effect on *Heterobasidion* hosts, Table 12. Fungal strain RK5A with HetPV2, HetRV6 and HetPV13 showed 22.1% decreased fungal growth. All three isolates of 7R18-11 with multiple viruses showed more than 100% increased fungal growth. Among the three virus combinations, HetPV2, HetPV4, HetPV13 and HetPV16 had the strongest increased effect on the growth of 7R18-11. Both isolates of fungal strain SB10.16 with multiple viruses showed increased fungal growth of less than 10%. Between the two virus combinations, HetPV2, HetRV6, HetPV13 and HetPV16 had the strongest increased effect on the growth of SB10.16. All

three isolates of EV0789 infected by multiple viruses showed a little increased fungal growth of less than 5%. Among the three virus combinations, viruses HetPV2, HetRV6, HetPV13 and HetPV16 had the strongest increased effect on the growth of EV0789. Overall, the transmission effect of multiple viruses can be debilitating or beneficial, or not so much different from previous growth rate.

When growth rate is calculated in 10-50 cm^2 zone, multiple virus infection also caused variable effect on *Heterobasidion* hosts as is shown in Table 13. Fungal strain RK5A with HetPV2, HetRV6 and HetPV13 showed 19.8% decreased fungal growth. All three isolates of 7R18-11 with multiple viruses showed more than 100% increased fungal growth. Among the three virus combinations, HetPV2, HetPV4, HetPV13 and HetPV16 had the strongest increased effect on the growth of 7R18-11. Both isolates of fungal strain SB10.16 with multiple viruses showed increased fungal growth of around 10%. Between the two virus combinations, HetPV2, HetRV6, HetPV13 and HetPV16 had the strongest increased effect on the growth of SB10.16. All three isolates of EV0789 infected by multiple viruses showed very little change of fungal growth. The change is either slightly increase (less than 5%) or slightly decrease (less than 1%). Among the three virus combinations, viruses HetPV2, HetRV6, HetPV13 and HetPV16 had the strongest increased effect on the growth of EV0789. Viruses HetPV2, HetRV6, HetPV13 had a slightly debilitating effect on the fungal growth. Overall, the transmission effect of multiple viruses in 10-50 cm^2 interval is very close to that in 10-40 cm^2.

Table 12 The difference of average growth rate between virus free and multiple viruses infected fungus strain from 10 cm^2 to 40 cm^2

Fungus strain with hosting viruses in bracket	Average virus-free growth rate (cm^2/day)	Average multiple viruses infected growth rate (cm^2/day)	Difference (cm^2/day)	%
RK5A (HetPV2, HetRV6, HetPV13)	9.3822	7.3096	-2.0726	-22.1%
7R18-11 (HetPV2, HetRV6, HetPV9, HetPV13)	4.3542	9.041	4.6868	107.6%
7R18-11(HetPV2, HetPV4, HetPV13, HetPV16)	4.3542	9.9398	5.5856	128.3%
7R18-11(HetPV2, HetPV4, HetRV6, HetPV13, HetPV16)	4.3542	9.161	4.8068	110.4%
SB10.16 (HetPV2, HetRV6, HetPV13)	9.3786	9.6166	0.238	2.5%
SB10.16(HetPV2, HetRV6, HetPV13, HetPV16)	9.3786	10.0734	0.6948	7.4%
EV0789(HetPV2, HetPV13, HetPV16)	9.3786	9.439	0.0606	0.64%
EV0789 (HetPV2, HetRV6, HetPV13)	9.3786	9.3856	0.007	0.07%
EV0789 (HetPV2, HetRV6, HetPV13, HetPV16)	9.3786	9.8062	0.4276	4.6%

Table 13 The difference of average growth rate between virus free and multiple viruses infected fungus strain from 10 cm^2 to 50 cm^2.

Fungus strain with hosting viruses in bracket	Average virus-free growth rate (cm^2/day)	Average multiple viruses infected growth rate (cm^2/day)	Difference (cm^2/day)	%
RK5A (HetPV2, HetRV6, HetPV13)	10.1076	8.1058	-2.0018	-19.8%
7R18-11 (HetPV2, HetRV6, HetPV9, HetPV13)	4.2456	9.4352	5.1896	122%
7R18-11(HetPV2, HetPV4, HetPV13, HetPV16)	4.2456	10.21	5.9644	140%
7R18-11(HetPV2, HetPV4, HetRV6, HetPV13, HetPV16)	4.2456	9.4056	5.16	122%
SB10.16 (HetPV2, HetRV6, HetPV13)	9.7212	10.472	0.7508	7.7%
SB10.16(HetPV2, HetRV6, HetPV13, HetPV16)	9.7212	10.706	0.9848	10.1%
EV0789(HetPV2, HetPV13, HetPV16)	9.9512	10.0564	0.1052	1.1%
EV0789 (HetPV2, HetRV6, HetPV13)	9.9512	9.9116	-0.0396	-0.4%
EV0789 (HetPV2, HetRV6, HetPV13, HetPV16)	9.9512	10.526	0.5748	5.8%

5 Discussion

5.1 More replicates of transmission may lead to more precise transmission result

It is clearly shown in Table 9 and Table 10 that even there were only two duplicate experiments conducted, the virus transmission results are not always the same. Some viruses were successfully transmitted to the new host in both duplicates, while some were only transmitted in one but not the other. This result enlightens us that by doing more and more duplicates, a more precise and reliable virus transmission rate can be reached by counting the chances of successful transmission.

5.2 Transmission between species is considerably less frequent than within species

Although only two duplicates were done in this research, it appears that transmission of multiple viruses is more likely to happen within species border (from *H. parviporum* to *H. parviporum*) than across species border (from *H. parviporum* to *H. annosum*). Whether this rule applies to other *Heterobasidion* fungus strains needs to be proved. Also, to prove this statement, more replicates of transmission experiment needs to be conducted. In this study, the success of virus transmission is based on anastomosis between *H. parviporum* and *H. parviporum* species and between *H. parviporum* and *H. annosum* species. Somatic incompatibility can restrict hyphal fusion between two heterokaryons (Korhonen & Stenlid 1998), however there are scientific reports of successful virus transmission between somatically incompatible fungal individuals (Ihrmark et al. 2002, Vainio et al. 2013). Virus transmission between two different *Heterobasidion* intersterility groups have been reported as well (Ihrmark et al. 2002, Vainio et al. 2010, Jurvansuu et al. 2014, Kashif et al. 2015), but no studies have revealed that virus transmission is more likely to happen within same species than across species borders. The reason behind this phenomenon is unknown. A possible guess would be that the hyphae forms between more distant fungal strains are more different, making it harder for the viruses to transfer.

5.3 Why some viruses were more easily transmitted than others

Besides the fact that viruses were more likely to be transmitted from *H. parviporum* to *H. parviporum* than from *H. parviporum* to *H. annosum*, in this study, some viruses showed higher transmission success rate compared to other viruses. The most easily transmitted ones are HetPV2 and HetPV13, HetPV16 ranks below, while HetPV7 was not transmitted at all. It is possible that HetPV7 had disappeared from the original host. However, no verifying experiment was done to testify this. It is hard to explain why some viruses were more easily transmitted than others. Although HetRV6 is reported to be the most common virus existing in *Heterobasidion* species (Vainio et al. 2012), its transmission rate is not the highest in this thesis. This discovery is not what was expected at first. Reasons behind different transmission rate may lie in the recipient selection as well as the virus selection. It is possible that some viruses are naturally more easily to be transmitted among the selected recipients and some are not. Another reason is there are only two replicates in this thesis. The replicates were not numerous enough to make a more accurate transmission rate analysis.

5.4 The transmission of more viruses does not mean more debilitating growth

This discover is opposite of the hypothesis of this thesis. From the growth rate results, we can see that some fungi were successfully infected by all five viruses, for example 7R18-11, but infection by multiple viruses did not necessarily mean slower growth rate, in this case, no debilitating effects took place at all. Even in the recipient isolates showing decreased growth due to multiple virus infection, more viruses infection does not mean more decreased growth effect. Maybe one or two of the five viruses play a main role in affecting fungus growth, but when other viruses that can increase the growth rate also enter the fungus, the situation changes and becomes more complicated and unpredictable. It is also possible that co-infecting viruses compete with each other in their host and therefore their ability to inhibit fungus growth becomes weakened due to their own not so good living condition.

5.5 The transmission can result in variable effect in fungus growth

Multiple viruses can affect fungus growth rate in many ways, not necessarily decreasing their growth rate, but also increasing their growth rate, or sometimes does not make much difference. This agrees with Hyder's (2013) research on single virus effect on *Heterobasidion*.

In the case of fungus strain RK5A, three-virus-infection causes debilitation in growth greatly. Sasaki et al. (2016) reported co-infection of *Rosellinia necatrix* megabirnavirus 2 (RnMBV2) and *Rosellinia necatrix* partitivirus 1 (RnPV1) caused hypovirulence on host fungus *Rosellinia necatrix*. The research results of Potgieter et al. (2013) showed that *Botrytis cinerea* CCg378 was coinfected by two mycoviruses and that the hypovirulence of *B. cinereal* CCg378 could be conferred by both mycoviruses. Although these results are only related to two mycoviruses infection, the infection effect is similar with three viruses' infection in this study.

In this study, on SB10.16, although the t- test show that the difference is statistically significant (Table 30), the difference in growth rate between the virus free and multiple viruses infected SB10.16 is not big, less than 10%. On EV0789, the infection of multiple viruses causes very little changes on their growth rate, the t-test also shows there are no significant difference most times except in one case when the fungal isolate was infected by HetPV2, HetRV6, HetPV13 and HetPV16, and the growth rate was calculated within 10-50 cm^2. Co-infection had no obvious effects on the fungal host *S. nivalis*, which was infected by three different mycoviruses (Wu et al. 2016). In Chu's study (2004), multiple virus infections also did not cause phenotypic effects on the host fungus *Fusarium graminearum*.

In this study, only on fungus strain 7R18-11, multiple viruses increase its growth rate greatly. But the virus-free isolate of 7R18-11 may have been degenerated, see discussion 5.7.

5.6 Which virus or viruses play the crucial role in affecting the fungal growth is unknown

Since this study focuses on multiple virus transmission, only recipients with three or more viruses were chosen to be experimented in the final growth rate experiments. Some fungus strains were also successfully transmitted by only one or two viruses, they were not included in the following experiments. However, it would be interesting to compare the growth rate of a single virus infected, two viruses infected and 3 or more viruses infected *Heterobasidion* fungus strain. This way it will be easier to see which virus plays a dominant role in deciding the growth rate of their host and how it affects their host growth. It will be also evident whether the number of viruses affect *Heterobasidion* growth rate.

5.7 The strain 7R18-11 may have somehow degenerated after the storage but before growth rate experiments

Transmission results indicated that in all four plates of 7R18-11, 7R18-11 got viruses from the donor successfully, and among which in three pairings it got multi-virus (three different viruses or more), so limited growth during transmission experiment could be because of multi-virus infection. However, following growth rate experiment showed virus infected isolate grew much faster than virus free isolate. No debilitation effects happened. That means the limited growth in transmission experiment may not happen because of virus infection but because of degeneration before transmission experiment. But Professor Jarkko Hantula checked the growth rate of virus free 7R18-11 using my storage and Eeva Vainio's storage later, and both isolates had a relatively normal (and similar) growth rate.

As is shown in Figure 11, it took 7R18-11 around 20 days to cover the whole plate and only took around 10 days for other fungus strains to cover the whole plate (4.5 and Appendix 4), which leads me to believe, 7R18-11 degenerated somehow after storage but before growth rate experiment. *Heterobasidion* strain 7R18-11, called 18-05 in the

paper of Vainio et al (2015b), was originally infected with HetPV2, but it was cured of the virus by temperature treatment in 2011 (hence name 7R18-11). This is just speculation, but the thermal treatment may have affected the fungus so that it is more prone to degeneration. As virus free 7R18-11 grew slower than its normal state because of degeneration, the effect of multiple virus infection might be biased due to its survival state. That is to say, the beneficial effect of multiple virus infection on 7R18-11 could be biased too.

5.8 The growth rate in nature might be different from the growth rate tested in laboratory

In this thesis the growth rate experiment was conducted on malt plates under 20 degrees. This is a very different condition compared to natural environment. Malt plate medium is quite different from tree stumps in nature or wood substrate. The temperature is high and stable in the growth rate experiment of this thesis, while in nature, temperature varies through season. It would be interesting to conduct the growth rate experiment in forest on tree stumps and compare the results done in laboratory. At last, any effect of multiple virus infection in lab has the possibility to amplify or escalate in nature because viruses have more time and space to grow. In laboratory, the growth medium is limited on each plate which leads to limited growth days and limited time for the viruses to affect their hosts. In nature, the viruses have more time to affect the growth of their hosts.

6 Conclusions

Using the particular donor and recipient fungi in this study, viruses HetPV2, HetPV13 were easier to be transmitted to a new host than other tested viruses. Among all the fungus strains infected by multiple viruses, HetPV2 and HetPV13 were found in all of them.

The virus composition is not as important as the fungus. Infection by more viruses does not bring more growth debilitation to the *Heterobasidion* fungus.

Multiple virus transmission happened more frequently between same *Heterobasidion* species, *H. parviporum* to *H. parviporum* in this study. More replicates of transmission experiment and more strains of *Heterobasidion* fungus need to be tested to prove this conclusion.

Based on my results, at 20 °C it is possible to use multiple viruses to control the growth of recipient RK5A. But multiple virus infection does not affect the growth rate of other recipients big enough or sometimes even increase the growth rate of their hosts. Overall, more experiments in terms of more *Heterobasidion* strains, more partitiviruses combinations and more conditions need to be conducted to acknowledge the influence of multiple partitiviruses infection and whether it is feasible to control *Heterobasidion* with multiple virus infections.

7 Acknowledgement

This work was carried out at the Natural Resources Institute Finland (Luke) in Vantaa. I would like to thank my supervisors Prof. Jarkko Hantula and D.Sc. Eeva Vainio for all their help, guidance and support that I have received while working on this master´s thesis. Many thanks to Rafiq Hyder, Eeva Vainio and Jaana Jurvansuu for teaching me in my summer training work in Luke. I also want to thank everyone at Luke who has helped me during this process and for the inspiring atmosphere, as I always felt welcome to work there. I want to thank my professor Paula Elomaa from University of Helsinki, Department of Agricultural and Forestry.

I am truly grateful to all my fellow students with whom I have shared amazing memories and experiences during the years of my studies at the University. I am grateful to my family and friends for mental support.

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Appendix

Appendix 1 Growth mediums used for the experiments:

2 % Malt extract agar

Agar 13,5 g

Malt extract 18,0 g

H_2O 900 ml

MOS

Orange serum agar 15 g

Malt extract 8 g

Dextrose 8 g

Agar 9 g

H_2O 1000 ml

Appendix 2 Solutions for RNA extraction

Lysis buffer

50 mM TrisHCl

50 mM EDTA

3 % SDS

1 % β-merkaptoethanol

PEG

Polyethylene glycol 6 g

5M NaCl 15ml

Sterile water 15 ml

Chloroform–isoamyl alcohol 24:1

Isoamyl alcohol 2 ml

Chloroform 48 ml

Phenol–chloroform–isoamyl alcohol 25: 24: 1

Phenol 40 ml

Chloroform-isoamyl alcohol 40 ml

Appendix 3 RNA concentrations and amounts used for cDNA synthesis

Table 14 RNA concentrations and amounts used for cDNA synthesis. "(1)" and "(2)" represent replicate 1 and replicate 2 in the transmission experiment.

Sample	NanoVue RNA concentration, ng/µl	Volume taken, µl	Amount of RNA, µg
H. parviporum (LAP 3.3.2) as donor:			
H. parviporum (RK5A) (1)	1128	1.77	2
H. parviporum (RK5A) (2)	253.2	7.89	2
H. parviporum (7R18-11) (1)	638	3.13	2
H. parviporum (7R18-11) (2)	367.2	5.45	2
H. parviporum (SB10.16) (1)	2498	0.8	2
H. parviporum (SB10.16) (2)	934	2.14	2
H. parviporum (EV0789) (1)	1174	1.7	2
H. parviporum (EV0789) (2)	1064	2	2
H. annosum (S49-5*) (1)	529.6	3.78	2
H. annosum (S49-5*) (2)	3623	0.552	2
H. annosum (03021) (1)	986.4	2.03	2
H. annosum (03021) (2)	615.2	3.25	2
H. annosum (HA5.31) (1)	1183	1.7	2
H. annosum (HA5.31) (2)	1068	1.87	2
H. annosum (T60-9) (1)	1160	1.73	2
H. annosum (T60-9) (2)	1006	1.99	2
H. annosum (93173) (1)	3018	0.66	2
H. annosum (93173) (2)	772	2.6	2
H. annosum (RKON1.60) (1)	682.8	3	2
H. annosum (RKON1.60) (2)	588.8	3.4	2
H. parviporum (7R242) as donor:			
H. parviporum (RK5A) (1)	2282	0.876	2
H. parviporum (RK5A) (2)	319.2	6.27	2
H. parviporum (7R18-11) (1)	1147	1.74	2
H. parviporum (7R18-11) (2)	400.4	5	2
H. parviporum (SB10.16) (1)	2506	0.8	2

H. parviporum (SB10.16) (2)	751.6	2.66	2
H. parviporum (EV0789) (1)	1104	1.81	2
H. parviporum (EV0789) (2)	356.8	5.6	2
H. annosum (S49-5*) (1)	613.2	3.26	2
H. annosum (S49-5*) (2)	212	9.43	2
H. annosum (03021) (1)	2506	0.798	2
H. annosum (03021) (2)	1071	1.87	2
H. annosum (HA5.31) (1)	610.4	3.28	2
H. annosum (HA5.31) (2)	284	7.04	2
H. annosum (T60-9) (1)	835.6	2.4	2
H. annosum (T60-9) (2)	902.4	2.2	2
H. annosum (93173) (1)	500.8	4	2
H. annosum (93173) (2)	781.2	2.56	2
H. annosum (RKON1.60) (1)	922.8	2.2	2
H. annosum (RKON1.60) (2)	642.4	3.11	2

Appendix 4 Total growth data of all five parallels

Table 15 The total growth data of all five parallel replicates in the growth rate experiment of virus free RK5A.

Growth days of virus free RK5A	Growth area of plate1 (cm^2)	Growth area of plate2 (cm^2)	Growth area of plate3 (cm^2)	Growth area of plate4 (cm^2)	Growth area of plate5 (cm^2)	Average (cm^2)	stdev
0	0	0	0	0	0	0.000	0.000
1	0.466	0.422	0.292	0	0	0.236	0.225
2	1.498	1.33	1.278	0.698	1.472	1.255	0.325
3	3.57	2.95	2.986	2.218	3.332	3.011	0.512
4	6.466	4.956	5.654	4.474	6.53	5.616	0.908
5	10.98	7.774	10.106	8.542	11.372	9.755	1.552
6	17.63	13.904	17.542	14.706	18.61	16.478	2.048
7	27.556	22.1	27.578	23.87	29.06	26.033	2.916
8	38.562	32.534	38.968	34.916	40.29	37.054	3.218
9	51.796	46.55	52.362	48.432	52.25	50.278	2.638
10	55	54.132	55	55	55	54.826	0.388
11	55	55	55	55	55	55.000	0.000

Table 16 The total growth data of all five parallel replicates in the growth rate experiment of three viruses (HetPV2, HetRV6, HetPV13) infected RK5A.

Growth days of RK5A with 3 viruses (HetPV2, HetRV6, HetPV13)	Growth area of plate1 (cm^2)	Growth area of plate2 (cm^2)	Growth area of plate3 (cm^2)	Growth area of plate4 (cm^2)	Growth area of plate5 (cm^2)	Average (cm^2)	stdev
0	0	0	0	0	0	0.000	0
1	0.316	0.318	0.364	0.312	0.372	0.336	0.029
2	0.316	1.11	0.364	0.312	0.372	0.495	0.345
3	2.554	2.16	2.596	2.716	2.484	2.502	0.209
4	4.876	4.592	5.114	5.23	4.592	4.881	0.293
5	7.556	7.816	7.806	7.904	7.628	7.742	0.144
6	11.488	12.346	12.564	12.408	11.708	12.103	0.474
7	16.12	18.074	18.182	18.058	16.098	17.306	1.094
8	23.014	25.406	26.082	25.618	21.968	24.418	1.814
9	34.22	36.818	34.684	34.96	28.492	33.835	3.146
10	44.706	48.428	44.546	45.768	40.04	44.698	3.032
11	55	55	52.806	53.916	51.394	53.623	1.542
12	55	55	55	55	55	55.000	0

Table 17 The total growth data of all five parallel replicates in the growth rate experiment of virus free 7R18-11.

Growth days of virus free 7R18-11	Growth area of plate1 (cm^2)	Growth area of plate2 (cm^2)	Growth area of plate3 (cm^2)	Growth area of plate4 (cm^2)	Growth area of plate5 (cm^2)	Average (cm^2)	stdev
0	0	0	0	0	0	0.000	0.000
1	0	0	0	0.348	0	0.070	0.156
2	1.088	1.216	1.1	1.054	0.898	1.071	0.114
3	2.19	2.298	2.262	2.072	1.786	2.122	0.207
4	3.588	3.778	3.964	3.436	3.212	3.596	0.292
5	5.1	5.15	5.798	4.532	4.848	5.086	0.468
6	7.418	7.058	8.674	6.802	6.568	7.304	0.828
7	9.643	9.552	11.474	9.796	9.022	9.897	0.928
8	12.188	12.452	14.71	12.366	11.558	12.655	1.201
9	16.418	15.956	18.95	15.56	14.886	16.354	1.556
10	19.882	19.526	22.964	19.418	18.468	20.052	1.710
11	24.954	23.562	28.73	23.752	23.24	24.848	2.265
12	29.578	29.052	33.256	29.316	27.828	29.806	2.042
13	34.232	33.288	38.786	32.94	32.946	34.438	2.487
14	39.252	38.638	45.07	39.092	40.708	40.552	2.642
15	43.752	45.042	48.37	43.07	44.224	44.892	2.073
17	49.96	51.704	53.02	50.156	51.29	51.226	1.245
20	55	55	55	55	55	55.000	0.000

Table 18 The total growth data of all five parallel replicates in the growth rate experiment of 4 viruses (HetPV2, HetRV6, HetPV9, HetPV13) infected 7R18-11.

Growth days of 7R18-11 with 4 viruses (HetPV2, HetRV6, HetPV9, HetPV13)	Growth area of plate1 (cm^2)	Growth area of plate2 (cm^2)	Growth area of plate3 (cm^2)	Growth area of plate4 (cm^2)	Growth area of plate5 (cm^2)	Average (cm^2)	stdev
0	0	0	0	0	0	0.000	0.000
1	0.336	0.342	0	0.426	0.33	0.287	0.165
2	1.26	1.196	0.78	1.264	1.09	1.118	0.202
3	3.164	2.722	2.524	3.296	2.672	2.876	0.335
4	6.27	5.78	5.526	6.566	5.454	5.919	0.483
5	10.482	9.224	9.576	9.704	8.954	9.588	0.580
6	16.816	14.942	14.942	15.392	14.984	15.415	0.806
7	25.14	23.138	23.12	23.35	22.904	23.530	0.914
8	35.902	34.44	33.588	34.494	33.612	34.407	0.942
9	47.9	46.364	45.866	47.618	45.812	46.712	0.985
10	55	53.904	55	55	55	54.781	0.490
11	55	55	55	55	55	55.000	0.000

Table 19 The total growth data of all five parallel replicates in the growth rate experiment of 4 viruses (HetPV2, HetPV4, HetPV13, HetPV16) infected 7R18-11.

Growth days of 7R18-11 with 4 viruses (HetPV2, HetPV4, HetPV13, HetPV16)	Growth area of plate1 (cm^2)	Growth area of plate2 (cm^2)	Growth area of plate3 (cm^2)	Growth area of plate4 (cm^2)	Growth area of plate5 (cm^2)	Average (cm^2)	stdev
0	0	0	0	0	0	0.000	0.000
1	0.232	0.36	0	0	0	0.118	0.168
2	0.806	1.408	0.686	0.78	0.424	0.821	0.361
3	1.864	2.706	1.606	1.52	1.416	1.822	0.521
4	3.718	5.17	2.932	3.202	2.396	3.484	1.057
5	6.294	9.514	4.72	5.452	4.09	6.014	2.123
6	11.416	16.364	9.272	9.304	7.442	10.760	3.434
7	19.086	26.328	15.204	16.232	12.304	17.831	5.333
8	30.396	39.654	24.39	26.058	19.642	28.028	7.553
9	43.158	50.448	37.362	39.39	31.442	40.360	7.054
10	52.936	55	50.542	50.01	44.18	50.534	4.073
11	55	55	54.722	55	52.972	54.539	0.884
12	55	55	55	55	55	55.000	0.000

Table 20 The total growth data of all five parallel replicates in the growth rate experiment of 4 viruses (HetPV2, HetPV4, HetRV6, HetPV13, HetPV16) infected 7R18-11.

Growth days of 7R18-11 with 5 viruses (HetPV2, HetPV4, HetRV6, HetPV13, HetPV16)	Growth area of plate1 (cm^2)	Growth area of plate2 (cm^2)	Growth area of plate3 (cm^2)	Growth area of plate4 (cm^2)	Growth area of plate5 (cm^2)	Average (cm^2)	stdev
0	0	0	0	0	0	0.000	0.000
1	0	0	0	0	0	0.000	0.000
2	0.692	0.558	0.496	0.704	0.712	0.632	0.099
3	1.702	1.462	1.408	1.78	1.618	1.594	0.157
4	2.91	2.586	2.508	2.95	2.894	2.770	0.206
5	4.368	4.534	4.102	4.886	4.864	4.551	0.334
6	8.264	7.394	6.382	8.538	8.076	7.731	0.864
7	13.734	11.53	9.504	13.354	13.568	12.338	1.815
8	21.608	18.13	14.294	21.544	21.658	19.447	3.250
9	32.216	29.376	21.864	33.154	32.748	29.872	4.715
10	44.184	40.796	31.608	44.574	45.526	41.338	5.724
11	51.932	50.456	44.196	54.544	54.024	51.030	4.158
12	55	54.94	52.638	55	55	54.516	1.050
13	55	55	55	55	55	55.000	0.000

Table 21 The total growth data of all five parallel replicates in the growth rate experiment of virus free SB10.16.

Growth days of virus free SB10.16	Growth area of plate1 (cm^2)	Growth area of plate2 (cm^2)	Growth area of plate3 (cm^2)	Growth area of plate4 (cm^2)	Growth area of plate5 (cm^2)	Average (cm^2)	stdev
0	0	0	0	0	0	0.000	0.000
1	0.52	0.552	0.374	0.406	0.678	0.506	0.122
2	2.224	2.168	2.158	1.568	2.762	2.176	0.423
3	6.204	6.14	5.75	4.87	6.822	5.957	0.719
4	10.698	10.46	10.32	9.14	11.558	10.435	0.869
5	18.08	16.702	16.432	14.97	18.086	16.854	1.301
6	26.212	25.824	26.506	23.99	27.954	26.097	1.427
7	39.232	37.998	39.626	36.346	40.48	38.736	1.608
8	49.334	49.38	52.058	49.36	50.42	50.110	1.182
9	55	55	55	55	55	55.000	0.000

Table 22 The total growth data of all five parallel replicates in the growth rate experiment of 3 viruses (HetPV2, HetRV6, HetPV13) infected SB10.16.

Growth days of 3 viruses (HetPV2, HetRV6, HetPV13) infected SB10.16	Growth area of plate1 (cm^2)	Growth area of plate2 (cm^2)	Growth area of plate3 (cm^2)	Growth area of plate4 (cm^2)	Growth area of plate5 (cm^2)	Average (cm^2)	stdev
0	0	0	0	0	0	0	0
1	0.572	0.624	0	0.516	0.374	0.4172	0.251204
2	2.406	2.41	1.59	2.124	1.956	2.0972	0.343187
3	6.542	6.728	5.658	6	6.17	6.2196	0.426758
4	11.342	11.624	10.7	10.788	11.96	11.2828	0.539214
5	18.166	18.918	16.716	17.51	19.26	18.114	1.034608
6	28.338	29.016	26.392	27.308	29.488	28.1084	1.261588
7	42.002	42.972	40.13	40.926	42.66	41.738	1.192429
8	54.14	54.538	53.84	54.376	54.42	54.2628	0.277083
9	55	55	55	55	55	55	0

Table 23 The total growth data of all five parallel replicates in the growth rate experiment of 4 viruses (HetPV2, HetRV6, HetPV13, HetPV16) infected SB10.16.

Growth days of 4 viruses (HetPV2, HetRV6, HetPV13, HetPV16) infected SB10.16	Growth area of plate1 (cm^2)	Growth area of plate2 (cm^2)	Growth area of plate3 (cm^2)	Growth area of plate4 (cm^2)	Growth area of plate5 (cm^2)	Average (cm^2)	stdev
0	0	0	0	0	0	0.000	0.000
1	0.458	0	0.5	0	0	0.192	0.263
2	2.212	1.74	1.914	1.7	1.384	1.790	0.304
3	5.92	4.826	5.67	4.926	4.428	5.154	0.620
4	11.174	9.926	10.362	9.648	8.88	9.998	0.850
5	18.444	16.656	17.192	16.728	15.296	16.863	1.132
6	28.486	26.532	26.768	26.25	24.378	26.483	1.465
7	41.67	41.644	41.084	39.838	37.592	40.366	1.719
8	53.922	54.376	52.026	53.122	51.378	52.965	1.258
9	55	55	55	55	55	55.000	0.000

Table 24 The total growth data of all five parallel replicates in the growth rate experiment of virus free EV0789.

Growth area of virus free EV0789	Growth area of plate1 (cm^2)	Growth area of plate2 (cm^2)	Growth area of plate3 (cm^2)	Growth area of plate4 (cm^2)	Growth area of plate5 (cm^2)	Average (cm^2)	stdev
0	0	0	0	0	0	0	0
1	0	0.398	0.492	0.388	1.084	0.4724	0.390595
2	1.256	1.882	2.042	1.906	2.864	1.99	0.575338
3	3.402	4.3	4.93	4.83	5.932	4.6788	0.926037
4	7.444	8.598	8.862	9.11	10.57	8.9168	1.122962
5	13.52	14.954	15.42	14.888	17.772	15.3108	1.547778
6	21.866	23.024	23.666	22.894	26.028	23.4956	1.555797
7	32.504	34.634	35.862	33.974	37.946	34.984	2.051666
8	45.444	47.838	48.496	48.718	49.87	48.0732	1.642485
9	55	55	55	55	55	55	0

Table 25 The total growth data of all five parallel replicates in the growth rate experiment of 3 viruses (HetPV2, HetPV13, HetPV16) infected EV0789.

Growth area of 3 viruses (HetPV2, HetPV13, HetPV16) infected EV0789	Growth area of plate1 (cm^2)	Growth area of plate2 (cm^2)	Growth area of plate3 (cm^2)	Growth area of plate4 (cm^2)	Growth area of plate5 (cm^2)	Average (cm^2)	stdev
0	0	0	0	0	0	0	0
1	0.35	0.418	0.57	0.7	0.612	0.53	0.143325
2	1.586	1.578	2.248	2.554	2.434	2.08	0.467508
3	4.818	4.952	6.568	6.848	6.266	5.8904	0.941787
4	9.814	9.942	12.194	11.572	10.86	10.8764	1.027375
5	16.26	16.57	20.004	17.634	17.96	17.6856	1.477315
6	25.004	25.452	30.018	27.334	27.782	27.118	2.008862
7	36.97	37.772	43.79	39.91	41.026	39.8936	2.716301
8	51.622	49.764	52.61	51.29	51.844	51.426	1.048301
9	55	55	55	55	55	55	0

Table 26 The total growth data of all five parallel replicates in the growth rate experiment of 3 viruses (HetPV2, HetRV6, HetPV13) infected EV0789.

Growth area of 3 viruses (HetPV2, HetRV6, HetPV13) infected EV0789	Growth area of plate1 (cm^2)	Growth area of plate2 (cm^2)	Growth area of plate3 (cm^2)	Growth area of plate4 (cm^2)	Growth area of plate5 (cm^2)	Average (cm^2)	stdev
0	0	0	0	0	0	0	0
1	0.308	0.322	0.396	0.524	0.334	0.3768	0.088889
2	1.138	1.332	1.404	1.598	1.324	1.3592	0.165787
3	3.798	4.064	4.076	5.052	4.344	4.2668	0.479532
4	7.866	7.998	7.924	10.038	7.986	8.3624	0.938177
5	13.05	13.296	13.896	15.938	14.416	14.1192	1.147474
6	20.35	20.462	21.274	22.946	22.302	21.4668	1.13794
7	31.324	31.33	33.334	34.406	33.74	32.8268	1.421608
8	45.772	45.578	45.928	47.22	48.54	46.6076	1.257911
9	55	55	55	55	55	55	0

Table 27 The total growth data of all five parallel replicates in the growth rate experiment of 4 viruses (HetPV2, HetRV6, HetPV13, HetPV16) infected EV0789.

Growth area of 4 viruses (HetPV2, HetRV6, HetPV13, HetPV16) infected EV0789	Growth area of plate1 (cm^2)	Growth area of plate2 (cm^2)	Growth area of plate3 (cm^2)	Growth area of plate4 (cm^2)	Growth area of plate5 (cm^2)	Average (cm^2)	stdev
0	0	0	0	0	0	0	0
1	0	0.378	0.324	0	0.384	0.2172	0.199648
2	1.134	2.232	1.522	1.002	1.87	1.552	0.510306
3	3.496	5.344	4.112	2.746	5.03	4.1456	1.072417
4	7.588	9.662	8.414	6.854	9.306	8.3648	1.168005
5	13.71	15.348	15.382	12.89	15.194	14.5048	1.1396
6	21.96	25.158	22.97	21.142	24.168	23.0796	1.622049
7	33.148	36.974	35.02	32.94	36.406	34.8976	1.836776
8	47.856	50.72	49.72	46.996	50.9	49.2384	1.741219
9	55	55	55	55	55	55	0

Appendix 5 Data of growth curves

Table 28 Average growth data of RK5A. Unit of growth area is cm^2. Day 0 is the day fungus strain was inoculated on the plates. The average growth area data in this table were the average number of 5 growth area values from five parallel growth rate experiments. The stdev is a measure of how widely values are dispersed from the average value (the mean).

Growth days of RK5A	Average growth area of virus free mycelium （cm^2）	Average growth area of three viruses (HetPV2, HetRV6, HetPV13) infected Mycelium (cm^2)	stdev of virus free RK5A	stdev of three viruses (HetPV2, HetRV6, HetPV13) infected RK5A
0	0	0	0.000	0.000
1	0.236	0.336	0.225	0.029
2	1.255	0.495	0.325	0.345
3	3.011	2.502	0.512	0.209
4	5.616	4.881	0.908	0.293
5	9.755	7.742	1.552	0.144
6	16.478	12.103	2.048	0.474
7	26.033	17.306	2.916	1.094
8	37.054	24.418	3.218	1.814
9	50.278	33.835	2.638	3.146
10	54.826	44.698	0.388	3.032
11	55	53.623	0.000	1.542
12	55	55	0.000	0.000

Table 29 Average growth data of 7R18-11. Unit of growth area is cm^2. Day 0 is the day fungus strain was inoculated on the plates. The average growth area data in this table were the average number of 5 growth area values from five parallel growth rate experiments. The stdev is a measure of how widely values are dispersed from the average value (the mean). Number in the brackets represents the virus name: 2=HetPV2, 4=HetPV4, 6=HetRV6, 9=HetPV9, 13=HetPV13, 16=HetPV16.

Growth days	Average growth area of virus free 7R18-11	Average growth area of 7R18-11 with 4 viruses (2,6,9, 13)	Average growth area of 7R18-11 with 4 viruses (2,4,13,16)	Average growth area of 7R18-11 with 5 viruses (2,4, 6,13, 16)	stedv of virus free 7R18-11	stdev of 7R18-11 with 4 viruses (2,6,9, 13)	stdev of 7R18-11 with 4 viruses (2,4, 13,16)	stdev of 7R18-11 with 5 viruses (2,4, 6,13,16)
0	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
1	0.070	0.287	0.118	0.000	0.156	0.165	0.168	0.000
2	1.071	1.118	0.821	0.632	0.114	0.202	0.361	0.099
3	2.122	2.876	1.822	1.594	0.207	0.335	0.521	0.157
4	3.596	5.919	3.484	2.770	0.292	0.483	1.057	0.206
5	5.086	9.588	6.014	4.551	0.468	0.580	2.123	0.334
6	7.304	15.415	10.760	7.731	0.828	0.806	3.434	0.864
7	9.897	23.530	17.831	12.338	0.928	0.914	5.333	1.815
8	12.655	34.407	28.028	19.447	1.201	0.942	7.553	3.250
9	16.354	46.712	40.360	29.872	1.556	0.985	7.054	4.715
10	20.052	54.781	50.534	41.338	1.710	0.490	4.073	5.724
11	24.848	55.000	54.539	51.030	2.265	0.000	0.884	4.158
12	29.806	55	55.000	54.516	2.042		0.000	1.050
13	34.438	55	55	55.000	2.487			0.000
14	40.552	55	55	55	2.642			
15	44.892	55	55	55	2.073			
17	51.226	55	55	55	1.245			
20	55.000	55	55	55	0.000			

Table 30 Average growth data of SB10.16. Unit of growth area is cm^2. Day 0 is the day fungus strain was inoculated on the plates. The average growth area data in this table were the average number of 5 growth area values from five parallel growth rate experiments. The stdev is a measure of how widely values are dispersed from the average value (the mean).

Growth days of SB10.16	Average growth area of virus free SB10.16	Average growth area of 3 viruses (HetPV2, HetRV6, HetPV13) infected SB10.16	Average growth area of 4 viruses (HetPV2, HetRV6, HetPV13, HetPV16) infected SB10.16	stdev of virus free SB10.16	stdev of SB10.16 with 3 viruses (HetPV2, HetRV6, HetPV13)	stdev of SB10.16 with 4 viruses (HetPV2, HetRV6, HetPV13, HetPV16)
0	0.000	0.000	0.000	0.000	0.000	0.000
1	0.506	0.417	0.192	0.122	0.251	0.263
2	2.176	2.097	1.790	0.423	0.343	0.304
3	5.957	6.220	5.154	0.719	0.427	0.620
4	10.435	11.283	9.998	0.869	0.539	0.850
5	16.854	18.114	16.863	1.301	1.035	1.132
6	26.097	28.108	26.483	1.427	1.262	1.465
7	38.736	41.738	40.366	1.608	1.192	1.719
8	50.110	54.263	52.965	1.182	0.277	1.258
9	55.000	55.000	55.000	0.000	0.000	0.000

Table 31 Average growth data of EV0789. Unit of growth area is cm^2. Growth day 0 is the day the fungus agars were inoculated on the malt plates. The average growth area data in this table were the average number of 5 growth area values from five parallel growth rate experiments. The standard deviation (stdev) is a measure of how widely values are dispersed from the average value (the mean).

Growth days of EV0789	Average growth area of virus free EV0789	Average growth area of EV0789 with 3 viruses (HetPV2, HetPV13, HetPV16)	Average growth area of EV0789 with 3 viruses (HetPV2, HetRV6, HetPV13)	Average growth area of EV0789 with 4 viruses (HetPV2, HetRV6, HetPV13, HetPV16)	stedv of virus free EV0789	stdev of EV0789 with 3 viruses (HetPV2, HetPV13, HetPV16)	stdev of EV0789 with 3 viruses (HetPV2, HetRV6, HetPV13)	stdev of EV0789 with 4 viruses (HetPV2, HetRV6, HetPV13, HetPV16)
0	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
1	0.472	0.530	0.377	0.217	0.391	0.143	0.089	0.200
2	1.990	2.080	1.359	1.552	0.575	0.468	0.166	0.510
3	4.679	5.890	4.267	4.146	0.926	0.942	0.480	1.072
4	8.917	10.876	8.362	8.365	1.123	1.027	0.938	1.168
5	15.311	17.686	14.119	14.505	1.548	1.477	1.147	1.140
6	23.496	27.118	21.467	23.080	1.556	2.009	1.138	1.622
7	34.984	39.894	32.827	34.898	2.052	2.716	1.422	1.837
8	48.073	51.426	46.608	49.238	1.642	1.048	1.258	1.741
9	55.000	55.000	55.000	55.000	0.000	0.000	0.000	0.000

Appendix 6 Data of growth rate

Table 32 Average logarithmic growth rate of RK5A. The average growth rate is the average value of five parallel growth rates, the stdev value is a measure of how widely values are dispersed from the average value (the mean).

RK5A	Average growth rate of virus free mycelium	Average growth rate of 3 viruses (HetPV2, HetRV6 and HetPV13) infected mycelium	stdev of virus free RK5A	stdev of 3 viruses infected RK5A
Growth rate (10-40 cm^2) cm^2/day	9.3822	7.3096	0.293028	0.872475
Growth rate (10-50 cm^2) cm^2/day	10.1076	8.1058	0.293198	0.297134

Table 33 Average logarithmic growth rate of 7R18-11. The average growth rate is the average value of five parallel growth rates, the stdev value is a measure of how widely values are dispersed from the average value (the mean).

Average growth rate of 7R18-11	Virus free mycelium	4 viruses (2, 6, 9, 13) infected mycelium	4 viruses (2, 4, 13, 16) infected mycelium	5 viruses (2, 4, 6, 13 ,16) infected mycelium	stdev of virus free mycelium	stdev of 4 viruses (2, 6, 9 ,13) infected mycelium	stdev of 4 viruses (2, 4, 13, 16) infected mycelium	stdev of 5 viruses (2, 4, 6, 13, 16) infected mycelium
Growth rate (10-40 cm^2)	4.3542	9.041	9.9398	9.161	0.186903	0.229528865	0.27819184	0.409252978
Growth rate (10-50 cm^2)	4.2456	9.4352	10.21	9.4056	0.206429	0.121594408	0.279427987	0.432202846

Table 34 Average logarithmic growth rate of SB10.16. The average growth rate is the average value of five parallel growth rates, the stdev value is a measure of how widely values are dispersed from the average value (the mean).

Average growth rate of SB10.16	Virus free mycelium	3 viruses (2, 6, 13) infected mycelium	4 viruses (2, 6, 13, 16) infected mycelium	stdev of virus free mycelium	stdev of 3 viruses (2, 6 ,13) infected mycelium	stdev of 4 viruses (2, 6, 13, 16) infected mycelium
Growth rate (10-40 cm^2)	9.3786	9.6166	10.0734	0.207241	0.135059	0.280821
Growth rate (10-50 cm^2)	9.7212	10.472	10.706	0.392825	0.120748	0.385795

Table 35 Average logarithmic growth rate of EV0789. Each average growth rate is the average value of five parallel growth rates, the stdev value is a measure of how widely values are dispersed from the average value (the mean).

Average growth rate of EV0789	Virus free mycelium	3 viruses (2, 13, 16) infected mycelium	3 viruses (2, 6, 13) infected mycelium	4 viruses (2, 6, 13,16) infected mycelium	stdev of virus free mycelium	stdev of 3 viruses (2, 13, 16) infected mycelium	stdev of 3 viruses (2, 6, 13) infected mycelium	stdev of 4 viruses (2, 6, 13, 16) infected mycelium
Growth rate (10-40 cm^2)	9.3786	9.439	9.3856	9.8062	0.207241	0.246264	0.34837	0.176914386
Growth rate (10-50 cm^2)	9.9512	10.0564	9.9116	10.526	0.10912	0.282978	0.358353	0

Appendix 7 Primary statistical analysis

Table 36 Growth rate comparison between virus free RK5A and 3 viruses (HetPV2, HetRV6 and HetPV13) infected RK5A including five parallels.

Plate number	Growth rate of virus free RK5A (10-40 cm^2) cm^2/day	Growth rate of 3 viruses (HetPV2, HetRV6 and HetPV13) infected RK5A (10-40 cm^2) cm^2/day	Growth rate of virus free RK5A (10-50 cm^2) cm^2/day	Growth rate of 3 viruses (HetPV2, HetRV6 and HetPV13) infected RK5A (10-50 cm^2) cm^2/day
Plate 1	9.091	7.692	10	8.163
Plate 2	9.677	7.895	10.256	8.511
Plate 3	9.677	7.5	10.526	8
Plate 4	9.375	7.692	10	8.163
Plate5	9.091	5.769	9.756	7.692

Table 37 Growth rate comparison between virus free 7R18-11 and 4 viruses (HetPV2, HetRV6, HetPV9, HetPV13) infected 7R18-11, including the growth rate of all five parallels.

Plate number	Growth rate of virus free 7R18-11(10-40 cm^2) cm^2/day	Growth rate of 4 viruses (HetPV2, HetRV6, HetPV9, HetPV13) infected 7R18-11 (10-40 cm^2) cm^2/day	Growth rate of virus free 7R18-11(10-50 cm^2) cm^2/day	Growth rate of 4 viruses (HetPV2, HetRV6, HetPV9, HetPV13) infected 7R18-11 (10-50 cm^2) cm2/day
Plate 1	4.225	8.824	4.04	9.302
Plate 2	4.286	9.375	4.396	9.524
Plate 3	4.478	8.824	4.396	9.302
Plate 4	4.167	9.091	4	9.524
Plate5	4.615	9.091	4.396	9.524

Table 38 Growth rate comparison between virus free 7R18-11 and 4 viruses (HetPV2, HetPV4, HetPV13, HetPV16) infected 7R18-11, including the growth rate of all five parallels.

Plate number	Growth rate of virus free 7R18-11(10-40 cm^2) cm^2/day	Growth rate of 4 viruses (HetPV2, HetPV4, HetPV13, HetPV16) infected 7R18-11 (10-40 cm^2) cm^2/day	Growth rate of virus free 7R18-11(10-50 cm^2) cm^2/day	Growth rate of 4 viruses (HetPV2, HetPV4, HetPV13, HetPV16) infected 7R18-11 (10-50 cm^2) cm^2/day
Plate 1	4.225	10	4.04	10.256
Plate 2	4.286	10.345	4.396	10.256
Plate 3	4.478	9.677	4.396	10.526
Plate 4	4.167	10	4	10.256
Plate5	4.615	9.677	4.396	9.756

Table 39 Growth rate comparison between virus free 7R18-11 and 5 viruses (HetPV2, HetPV4, HetRV6, HetPV13, HetPV16) infected 7R18-11, including the growth rate of all five parallels.

Plate number	Growth rate of virus free 7R18-11(10-40 cm^2) cm^2/day	Growth rate of 5 viruses (HetPV2, HetPV4, HetRV6, HetPV13, HetPV16) infected 7R18-11 (10-40 cm^2) cm^2/day	Growth rate of virus free 7R18-11(10-50 cm^2) cm^2/day	Growth rate of 5 viruses (HetPV2, HetPV4, HetRV6, HetPV13, HetPV16) infected 7R18-11 (10-50 cm^2) cm^2/day
Plate 1	4.225	9.091	4.04	9.091
Plate 2	4.286	9.375	4.396	9.524
Plate 3	4.478	8.571	4.396	8.889
Plate 4	4.167	9.091	4	9.524
Plate5	4.615	9.677	4.396	10

Table 40 Growth rate comparison between virus free SB10.16 and 3 viruses (HetPV2, HetRV6, HetPV13) infected SB10.16, including the growth rate of all five parallels.

Plate number	Growth rate of virus free SB10.16 (10-40 cm^2) cm^2/day	Growth rate of 3 viruses (HetPV2, HetRV6, HetPV13) infected SB10.16 (10-40 cm^2) cm^2/day	Growth rate of virus free SB10.16 (10-50 cm^2) cm^2/day	Growth rate of 3 viruses (HetPV2, HetRV6, HetPV13) infected SB10.16 (10-50 cm^2) cm^2/day
Plate 1	9.375	9.677	9.524	10.256
Plate 2	9.375	9.677	9.524	10.526
Plate 3	9.677	9.375	10.256	10.526
Plate 4	9.375	9.677	10	10.526
Plate5	9.091	9.677	9.302	10.526

Table 41 Growth rate comparison between virus free SB10.16 and 4 viruses (HetPV2, HetRV6, HetPV13, HetPV16) infected SB10.16, including the growth rate of all five parallels.

Plate number	Growth rate of virus free SB10.16 (10-40 cm^2) cm^2/day	Growth rate of 4 viruses (HetPV2, HetRV6, HetPV13, HetPV16) infected SB10.16 (10-40 cm^2) cm^2/day	Growth rate of virus free SB10.16 (10-50 cm^2) cm^2/day	Growth rate of 4 viruses (HetPV2, HetRV6, HetPV13, HetPV16) infected SB10.16 (10-50 cm^2) cm^2/day
Plate 1	9.375	9.677	9.524	10.526
Plate 2	9.375	10.345	9.524	11.111
Plate 3	9.677	10	10.256	10.256
Plate 4	9.375	10.345	10	11.111
Plate5	9.091	10	9.302	10.526

Table 42 Growth rate comparison between virus free EV0789 and 3 viruses (HetPV2, HetPV13, HetPV16) infected EV0789, including the growth rate of all five parallels.

Plate number	Growth rate of virus free EV0789 (10-40 cm^2) cm^2/day	Growth rate of 3 viruses (HetPV2, HetPV13, HetPV16) infected EV0789 (10-40 cm^2) cm^2/day	Growth rate of virus free EV0789 (10-50 cm^2) cm^2/day	Growth rate of 3 viruses (HetPV2, HetPV13, HetPV16) infected EV0789 (10-50 cm^2) cm^2/day
Plate 1	9.375	9.375	10	10.526
Plate 2	9.375	9.375	10	10
Plate 3	9.677	9.677	10	10
Plate 4	9.091	9.091	10	9.756
Plate5	9.375	9.677	9.756	10

Table 43 Growth rate comparison between virus free EV0789 and 3 viruses (HetPV2, HetRV6, HetPV13) infected EV0789, including the growth rate of all five parallels.

Plate number	Growth rate of virus free EV0789 (10-40 cm^2) cm^2/day	Growth rate of 3 viruses (HetPV2, HetRV6, HetPV13) infected EV0789 (10-40 cm^2) cm^2/day	Growth rate of virus free EV0789 (10-50 cm^2) cm^2/day	Growth rate of 3 viruses (HetPV2, HetRV6, HetPV13) infected EV0789 (10-50 cm^2) cm^2/day
Plate 1	9.375	9.375	10	10
Plate 2	9.375	9.375	10	10
Plate 3	9.677	9.677	10	10
Plate 4	9.091	8.824	10	9.302
Plate5	9.375	9.677	9.756	10.256

Table 44 Growth rate comparison between virus free EV0789 and 4 viruses (HetPV2, HetRV6, HetPV13, HetPV16) infected EV0789, including the growth rate of all five parallels.

Plate number	Growth rate of virus free EV0789 (10-40 cm^2) cm^2/day	Growth rate of 4 viruses (HetPV2, HetRV6, HetPV13, HetPV16) infected EV0789 (10-40 cm^2) cm^2/day	Growth rate of virus free EV0789 (10-50 cm^2) cm^2/day	Growth rate of 4 viruses (HetPV2, HetRV6, HetPV13, HetPV16) infected EV0789 (10-50 cm^2) cm^2/day
Plate 1	9.375	10	10	10.526
Plate 2	9.375	9.677	10	10.526
Plate 3	9.677	9.677	10	10.526
Plate 4	9.091	10	10	10.526
Plate5	9.375	9.677	9.756	10.526

Table 45 p value's output of all comparisons

Variable 1	Variable 2	Growth rate calculated zone	p(T<=t) two tail	Compared with 0.05	Compared with 0.01	Compared with 0.001	Asterisks
Growth rate of virus free RK5A	Growth rate of three viruses (HetPV2, HetRV6 and HetPV13) infected RK5A	10-40 cm^2	0.003518365	<0.05	<0.01	>0.001	**
		10-50 cm^2	0.000143847	<0.05	<0.01	<0.001	***
Growth rate of Virus free 7R18-11	Growth rate of 4 viruses (HetPV2, HetRV6, HetPV9, HetPV13) infected 7R18-11	10-40 cm^2	4.61E-06	<0.05	<0.01	<0.001	***
		10-50 cm^2	8.68E-07	<0.05	<0.01	<0.001	***
Growth rate of Virus free 7R18-11	Growth rate of 4 viruses (HetPV2, HetPV4, HetPV13, HetPV16) infected 7R18-11	10-40 cm^2	8.48E-06	<0.05	<0.01	<0.001	***
		10-50 cm^2	3.59E-06	<0.05	<0.01	<0.001	***
Growth rate of Virus free 7R18-11	Growth rate of 5 viruses (HetPV2, HetPV4, HetRV6, HetPV13, HetPV16) infected 7R18-11	10-40 cm^2	1.25E-05	<0.05	<0.01	<0.001	***
		10-50 cm^2	1.3E-05	<0.05	<0.01	<0.001	***

Growth rate of virus free SB10.16	Growth rate of 3 viruses (HetPV2, HetRV6, HetPV13) infected SB10.16	10-40 cm^2	0.177873	>0.05	>0.01	>0.001	
		10-50 cm^2	0.011238	<0.05	>0.01	>0.001	*
Growth rate of virus free SB10.16	Growth rate of 4 viruses (HetPV2, HetRV6, HetPV13, HetPV16) infected SB10.16	10-40 cm^2	0.011339	<0.05	>0.01	>0.001	*
		10-50 cm^2	0.020576	<0.05	>0.01	>0.001	*
Growth rate of virus free EV0789	Growth rate of 3 viruses (HetPV2, HetPV13, HetPV16) infected EV0789	10-40 cm^2	0.373901	>0.05	>0.01	>0.001	
		10-50 cm^2	0.465246	>0.05	>0.01	>0.001	
Growth rate of virus free EV0789	Growth rate of 3 viruses (HetPV2, HetRV6, HetPV13) infected EV0789	10-40 cm^2	0.941784	>0.05	>0.01	>0.001	
		10-50 cm^2	0.845853	>0.05	>0.01	>0.001	
Growth rate of virus free EV0789	Growth rate of 4 viruses (HetPV2, HetRV6,	10-40 cm^2	0.051608	>0.05	>0.01	>0.001	

	HetPV13, HetPV16) infected EV0789	10-50 cm^2	0.000297	<0.05	<0.01	<0.001	***